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5 mM) but not to $MgCl_2$. The WBB06 *eptB::kan* construct was able grow in Ca^{++}-containing medium when functional EptB was supplied *in trans* from a plasmid harboring *eptB* under *lac* promoter control, eliminating the possibility of polar effects. These results show that the modification of the outer Kdo moiety of LPS with pEtN is essential for viability of heptose-deficient *E. coli* cells grown in the presence of 5-50 mM Ca^{++}, possibly by modulating the affinity of LPS for Ca^{++}. Supported by NIH grant GM-51310 to C. R. H. Raetz.

368.2

Protein-protein interactions between acyl carrier protein (ACP) and β-ketoacyl-ACP reductase (FabG)

Yong-Mei Zhang, Charles O. Rock. Dept. of Infectious Diseases, St. Jude Children's Research Hospital, 332 N. Lauderdale St., Memphis, TN 38105

Fatty acid synthesis in bacteria is catalyzed by a set of individual enzymes (FAS II) which all interact with acyl carrier protein (ACP). However, the catalytic enzymes do not possess a primary sequence similarity that would indicate a universal binding motif for ACP. In contrast, ACP from all species have sequence similarity along helix II downstream of the prosthetic group attachement site. Our study utilizes the FAS II enzyme β-ketoacyl-ACP reductase (FabG) to validate the hypothesis that the "recognition helix" of ACP binds to a constellation of Arg residues adjacent to the entrance of the active site cavity. FabG mutants of Arg129 and Arg172 at the proposed ACP-binding site were created by site-directed mutagenesis. The activities of the mutants were assessed using both an ACP-dependent and an ACP-independent assay. Both single mutants and the double mutant exhibited impaired activity in the ACP-dependent assay, but the mutations did not affect activity in the ACP-independent assay. Direct binding studies using BIACORE and AlphaScreen technology confirmed that the FabG mutants lost the ability to bind ACP in comparison to the wild type protein. (Supported by GM34496 and ALSAC)

368.3

Characterization of GP-PDE1/MIR16, a member of mammalian Glycerophosphodiester Phosphodiesterase Family

bin zheng[1], christopher berrie[2], Daniela Corda[2], Marilyn Farquhar[1]. [1]UCSD, 9500 Gilman Dr., La jolla, CA 92093, [2]CMNS, Chieti, Italy

During the breakdown of phosphoinositides, glycerophosphoinositols are hydrolyzed to glycerol-3-phosphates and inositols by a glycerophosphoinositol phosphodiesterase (GPI-PDE). However, the protein responsible for this activity in mammals has not been identified to date. Previously we have identified MIR16 (Membrane interacting protein of RGS16) as an integral membrane glycoprotein that interacts with RGS proteins. Here we show that MIR16 belongs to a large family of glycerophosphodiester phosphodiesterases (GP-PDEs), whose signature is a conserved putative catalytic GP-PDE domain that shares a common sequence motif with the catalytic domains of mammalian PLCs. Expression of wild-type MIR16 (renamed as GP-PDE1), but not two mutants with mutations in the GP-PDE domain, in HEK293 cells lead to a dramatic increase in GPI-PDE activity. Analysis of substrate specificity showed that GP-PDE1 selectively hydrolyzes GPI. Membrane topology studies suggest that the N-terminal catalytic GP-PDE domain of MIR16 faces the lumen and the C-terminus faces the cytoplasm. Furthermore, the GPI-PDE activity of GP-PDE1 expressed in HEK293 cells is regulated by stimulation of several G protein-coupled receptors tested. Our results suggest that GP-PDE1 is a GPI-PDE that may participate in G protein signaling transduction and the regulation of phosphoinositide metabolism.

368.4

Purification and Characterization of Fatty Acid Transport Protein 1

Angela Marie Hall, David A. Hall. Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 420 Wahington Ave SE, Minneapolis, MN 55455

The Fatty Acid Transport Protein (FATP) family members have been implicated as bifunctional proteins involved in lipid metabolism, functioning in both the cellular uptake of fatty acids and the conversion of fatty acids to fatty acyl coenzyme A (CoA) esters. Overexpression of FATP1 in HEK 293 cells results in an increased rate of fatty acid influx for both C18:1 and C24:0 (long and very long chain fatty acids; Hatch, et al. J Lipid Res. 2002) that are channeled to triacylglycerol biosynthesis. Previous work has characterized FATP1 as a very long chain acyl CoA synthetase; here we demonstrate that FATP1 acyl CoA synthetase activity has broad substrate specificity toward both long and very long chain fatty acids.

To examine the kinetic properties of mmFATP1 acyl CoA synthetase activity, the murine FATP1 cDNA was subcloned in a 6X Histidine-tag vector and overexpressed in COS1 cells. Purification of His-tagged mmFATP1 by nickel affinity chromatography resulted in homogeneous protein with a specific activity of 2 μmol/min/mg for C24:0 and 1.2 μmol/min/mg for C16:0. Enzymatic conversion of C24:0 by FATP1 revealed a $V_{max\ apparent}$ of 1 nmole/min with a $K_{m\ apparent}$ of 12 μM while esterification of C16:0 by FATP1 resulted in a $V_{max\ apparent}$ of 0.6 nmole/min with a $K_{m\ apparent}$ of 23 μM. This *in vitro* data indicates that FATP1 is an acyl CoA synthetase with broad specificity for both long and very long chain fatty acids and supports the hypothesis that mmFATP1 facilitates the influx of fatty acids into adipocytes as well as skeletal and cardiac muscle by vectoral acylation. Supported by grants from the NIH and NSF.

368.5

Mechanism by which Phospholipase A_2 Causes Cells to Become Resistant to its Action

Heather A. Wilson[1], Allan M. Judd[2], John D. Bell[2]. [1]Biology, Utah Valley State College, 800 West University Parkway, Orem, Utah 84057, [2]Physiology and Developmental Biology, Brigham Young University, Provo, UT

Incubation of cells with extracellular secretory phospholipase A_2 (PLA_2) causes their plasma membrane to become refractory to further hydrolysis by additional enzyme. The objective of this study was to identify possible mechanisms for this phenomenon. Refractoriness displayed a rapid onset (half time of 13 s), was fully reversible, and was independent of membrane hydrolysis by the enzyme. Phospholipase C (PLC) also rendered cells resistant to hydrolysis by PLA_2, while phospholipase D was unable to induce refractoriness. Kinetic experiments and measurements of the rate of phospholipid extraction by albumin in human erythrocytes implied that refractoriness interferes with the migration of substrate molecules into the active site of bound PLA_2 rather than disrupting the ability of the enzyme to adsorb to the membrane surface. Two-photon scanning microscopy of erythrocytes labeled with a fluorescent probe of membrane physical properties, laurdan, suggested that PLA_2 hydrolyzes cell membranes at the interface between domains of differential fluidity. Incubation with PLC appeared to impair the formation of such boundaries. Based on these observations, we conclude that refractoriness is a result of biophysical changes to membrane structure that interfere with the ability of phospholipids to move into the active site of PLA_2 bound to the surface of the cell membrane.

368.6

Choline kinase has a protein kinase fold

Daniel Peisach, Pat Watanabe, Claudia Kent, Zhaohui Xu. Department of Biological Chemistry, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109-0606

Phosphatidylcholine (PC) is both a structural component of cellular membranes and a source component for several lipid messengers. The major pathway for PC synthesis is the CDPcholine pathway. Choline kinase (CK) catalyzes the first committed step in this pathway. Despite many years of research on this important enzyme, key questions remain unanswered including: 1) What is the mechanism of phospho-transfer and which specific protein residues contribute to catalysis? 2) How is choline kinase activity regulated? To address these questions and others, we solved the crystal structure of a 49 kDa choline kinase from *C. elegans*. The overall fold of CK has remarkable similarity to protein kinases despite limited sequence homology. Structural comparisons to protein kinases suggest that the ATP and choline binding sites are located in a space between the highly conserved portions of the N-

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Lipid biosynthetic genes and a ribosomal protein gene are cotranscribed

Sergey Podkovyrov, Timothy J. Larson*

Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0308, USA

Received 27 May 1995; revised version received 12 June 1995

Abstract **By using insertional mutagenesis we demonstrated that the *rpmF* gene encoding ribosomal protein L32, the *plsX* gene encoding a protein involved in membrane lipid synthesis and several fatty acid biosynthetic genes (*fabH*, *fabD* and *fabG*) are cotranscribed. Organization of these genes into an operon may play a role in the coordinate regulation of the synthesis of ribosomes and the cell membranes.**

Key words: *plsX* gene; Lipid biosynthetic gene; Ribosomal protein gene; Cotranscription; Insertional mutagenesis; *Escherichia coli*

1. Introduction

The rate of ribosome production in *Escherichia coli* is controlled in relation to bacterial growth rate (for review, see [1]). The synthesis rates of ribosomal proteins and rRNAs are strictly regulated so that the pools of free ribosomal components are small. Genes for the 52 ribosomal proteins are organized into at least 20 operons. Many of them contain genes for essential cellular processes including protein secretion, DNA replication, transcription and translation. The organization of these genes and the ribosomal protein genes into polycistronic transcription units is related to their coordinate regulation.

Recently we established the physical locations of genes surrounding the *plsX* gene of *E. coli* which encodes a protein involved in membrane lipid synthesis [2]. The *rpmF* gene encoding ribosomal protein L32 is located just upstream of the *plsX* gene and several fatty acid biosynthetic (*fab*) genes are located just downstream of the *plsX* gene (Fig. 1). Northern and promoter activity analysis suggested that the *rpmF-plsX-fab* genes comprise an operon (Oh and Larson, manuscript in preparation). In the present study, the effect of polar insertions into different sites of the *rpmF-plsX-fab* region was used to demonstrate cotranscription of the *rpmF*, *plsX*, *fabH*, *fabD* and *fabG* genes.

2. Materials and methods

Escherichia coli K-12 strain DH5αF'[F' φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_k^-,m_k^+) *supE44* λ^- *thi-1 gyrA96 relA1*] (Gibco BRL, Gaithersburg, MD, USA) was used as the host for DNA manipulations. Plasmid pSP417 [3] was used as the vector for construction of operon fusions and plasmid pHP45Ω [4] was the source of the spectinomycin omega cassette. As a source of DNA containing different parts of the *rpmF-plsX-fab* region we used an extensive plasmid collection generated in our laboratory. For plasmid DNA purification, Wizard Minipreps DNA Purification System was employed (Promega, Madison, WI, USA). DNA fragments for cloning were isolated from agarose gel by using Wizard PCR Preps DNA Purification System (Promega). All other standard molecular biology techniques were used, as described elsewhere [5]. β-Galactosidase activity encoded by the various *lacZ* fusions was assayed as described by Miller [6]. β-Galactosidase activity was measured at least in triplicate and the results given are the average of these data.

The complete nucleotide sequence of the *rpmF-plsX-fab* region was compiled from a number of sequences deposited in GenBank (for the accession numbers see [2]). Mapping of the restriction sites was carried out by using PC/GENE computer program [7].

3. Results and discussion

In order to determine which genes of the *rpmF-plsX-fab* region are cotranscribed we constructed a series of transcriptional fusions between different parts of the region and *lacZ* in the plasmid vector pSP417 designed for construction of transcriptional fusions. Then, the interposon Ω carrying a spectinomycin resistance gene (Sp') flanked by transcriptional termination signals in inverted orientations was inserted into different positions of the fusions. Strain DH5αF' was transformed with the recombinant plasmids, and the level of *lacZ* expression was measured. The structure of each fusion and corresponding β-galactosidase activity are summarized in Fig. 1.

To determine if *rpmF* and *plsX* are cotranscribed, the *SalI–SspI* DNA fragment containing the *g30k* gene for a 30-kDa protein with unknown function, the *rpmF* gene and the 5' part of the *plsX* gene was inserted into pSP417, yielding plasmid pSP419. This fragment was chosen for construction of the fusion because we recently showed that the *rpmF* gene is transcribed from the three promoters downstream of the *SalI* site located within coding and non-coding parts of *g30k* (manuscript in preparation). Expression of *lacZ* from recombinant plasmid pSP419 was compared to that obtained from the same plasmid with an Ω cassette inserted at the unique *Hin*dIII site just downstream of *rpmF* and 61 bp upstream of the *plsX* start codon (plasmid pSP422). Cotranscription of *rpmF* and *plsX* was indicated since *lacZ* expression was abolished in the case of pSP422. Although the mechanism of PlsX action is not established, it is known that the *plsX50* mutation together with *plsB26* encoding a defective *sn*-glycerol-3-phosphate acyltransferase is required for conferral of a glycerol-3-phosphate-auxotrophic phenotype [8]. Since *sn*-glycerol-3-phosphate acyltransferase catalyzes the initial reaction of membrane phospholipid synthesis in *E. coli*, PlsX may play an important role in the whole process. Cotranscription of *plsX* and *rpmF* may suggest coordinate regulation of the synthesis of ribosomes and membranes.

To find out if the *fabH* gene following the *plsX* gene is cotranscribed together with *rpmF* and *plsX*, insertional mutagenesis of the *g30k-rpmF-plsX-fabH-lacZ* fusion (plasmid pSP418) was performed. Ω insertion at the *Hin*dIII site upstream of the *plsX* gene decreased, but did not abolish *lacZ*

*Corresponding author. Fax: (1) (703) 231-9070.

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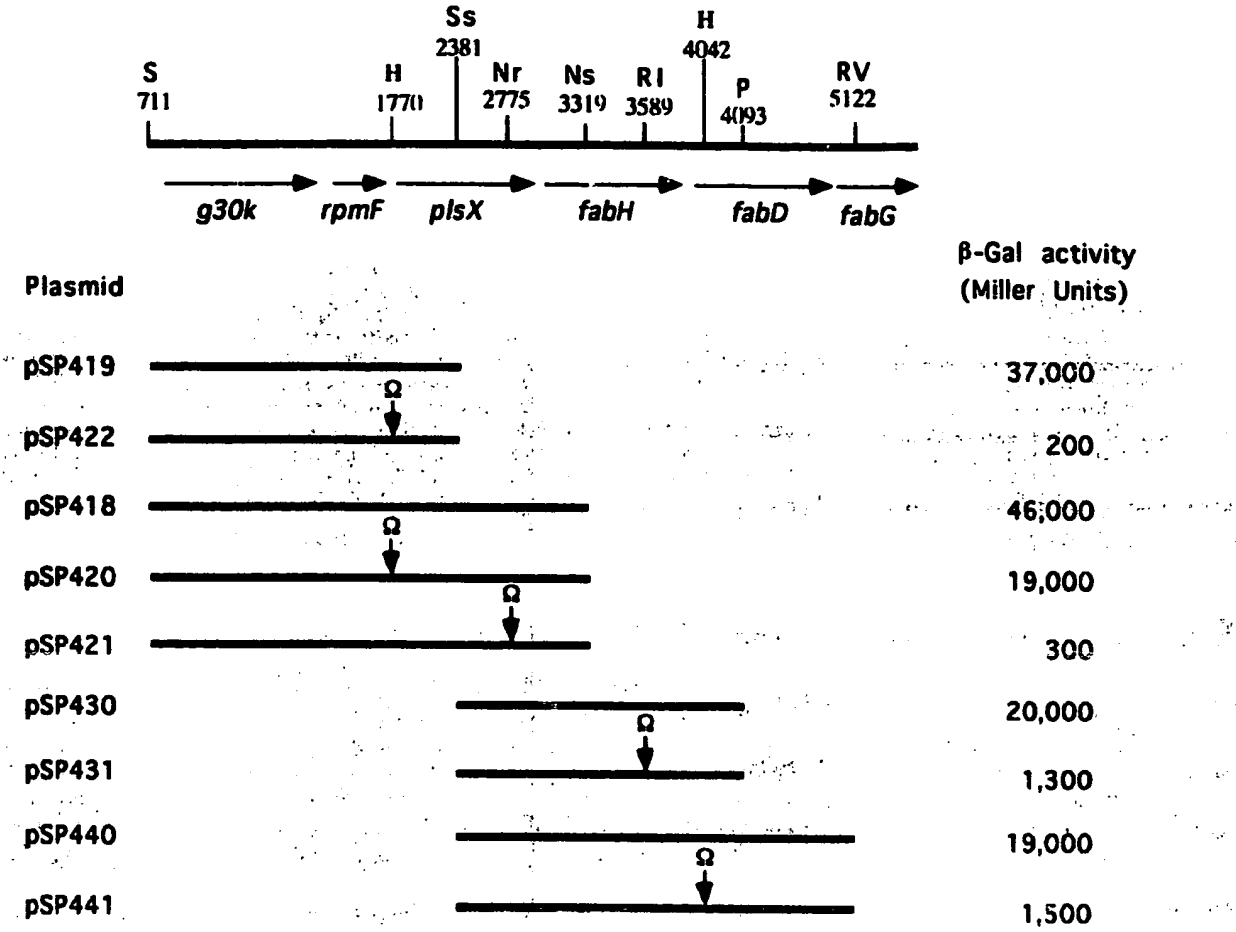

Fig. 1. Structure and analysis of transcriptional fusions. The indicated restriction fragments were cloned upstream of the promoterless *lacZ* gene of pSP417. Ω denotes the spectinomycin resistance omega cassette containing transcriptional terminators. DH5αF' cells were transformed with plasmids carrying the fusions and β-galactosidase activity was measured as described in section 2. Background β-galactosidase activity for DH5αF'(pSP417) was 50 U. Numbering of nucleotides starts from the first base of the *Pst*I site located within the *orfX* gene [2]. Restriction sites are abbreviated as follows: S, *Sal*I; H, *Hin*dIII; Ss, *Ssp*I; Nr, *Nru*I; Ns, *Nsi*I; RI, *Eco*RI; P, *Pvu*II; RV, *Eco*RV. Only those restriction sites used for cloning or insertional mutagenesis are indicated.

expression (plasmid pSP420) while the insertion within the 3' part of the *plsX* gene at *Nru*I abolished expression of *lacZ* (plasmid pSP421). These results indicate the presence of an additional promoter within the *plsX* gene that contributes to *fabH* transcription. The extent of the polar effect revealed that this promoter, in multicopy plasmids, provides approximately 40% of the *fabH* transcription.

Similar insertional mutagenesis was performed for *fabH* and *fabD* (plasmids pSP430 and pSP431) and for *fabD* and *fabG* (pSP440 and pSP441). The strong polar effects of insertions at either the *Eco*RI site (plasmid pSP431) or the *Hin*dIII site (plasmid pSP441) showed that the *fabH* transcripts continue into *fabD* and *fabG* and all three genes are cotranscribed. *fabH* encodes β-ketoacyl-ACP synthase III that may be a potential regulator of fatty acid biosynthesis in bacteria [9]. Malonyl CoA-ACP transacylase encoded by *fabD* provides malonyl-ACP, the key intermediate of fatty acid synthesis [10]. Mutants deficient in malonyl CoA-ACP transacylase require both saturated and unsaturated fatty acids for growth [11]. *fabG* encodes 3-ketoacyl-ACP reductase acting on an elongation step of fatty acid biosynthesis [10].

Based on the results of analysis of all the fusions shown in Fig. 1, we concluded that the *rpmF* gene and the *plsX-fab* genes are cotranscribed. This is the only known example where lipid biosynthetic genes and a ribosomal protein gene comprise an operon. Such organization is likely to play an important role in the coordinate regulation of ribosome and cell membrane synthesis. Further studies concerning transcriptional organization and regulation of the *rpmF-plsX-fab* operon are in progress.

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of how this pathogenic bacterium contributes to the disease state of afflicted individuals, we have used high-density DNA microarrays, consisting of unique human cDNA clones, to monitor gene expression in A549 lung epithelial tissue cultures during infection with *P. aeruginosa*. The mRNA transcripts isolated from A549 cells that were exposed to either wild-type bacteria or an isogenic mutant were used to synthesize cDNA probes labelled with either Cy3 or Cy5 fluorescent dye. The probes were combined and hybridized to a single microarray, which normalizes for differential hybridizations. The microarray was scanned using a laser scanner and a false-color computer image is produced for analysis. Using custom array analysis software (E. Hammersmark and R. Bumgarner, University of Washington), we have identified a set genes which are differentially regulated upon infection, several of which require interaction with *P. aeruginosa* and the expression of specific bacterial products. Although these genes are involved in a variety of functions, a number are involved in immune and inflammatory response signalling pathways. By comparing expression patterns during infection with different mutants, we have identified several genes which may play an important role in *P. aeruginosa* pathogenesis.

D-149. Development of New Tools to Facilitate Genetic Manipulations in *Pseudomonas aeruginosa*

L. A. Silo-Suh, S. J. Suh, D. E. Ohman
Virginia Commonwealth University and
McGuire VA Medical Center, Richmond, VA

To facilitate the study of the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, we developed several molecular tools for use in this bacterium. These include a tightly regulated promoter/repressor system to control gene expression, a temperature-sensitive replicon, and a series of cassettes carrying either *oriT* of RP4 plasmid, the *P. aeruginosa* stabilizing fragment (SF), or the tetracycline resistance gene (Tc^r). To control gene expression in *P. aeruginosa*, an integrating-plasmid was developed that carried the T7(A1/04/03) promoter, the $lacI^q$ gene for repression, multiple cloning sites for cloning convenience, and the gentamicin resistance marker for selection of recombinants in *P. aeruginosa*. We have used this system to regulate the expression of *rhlR*, a cell density dependent global gene regulator. Another tool developed here was a temperature-sensitive replicon that can provide a complementing gene that can later be eliminated. A commonly used *P. aeruginosa* replicon is on a 1.9 kb *Pst*I fragment called the *P. aeruginosa* stabilizing fragment (SF). However, it is normally very stable in *P. aeruginosa* and difficult to cure. To overcome this property, we isolated a temperature-sensitive *P. aeruginosa* replicon by random PCR mutagenesis of SF and screened for one that was unstable at 42°C in *P. aeruginosa*. Following growth at 42°C for 12-15 h, most of the cells lose the mutant plasmid. We have used this SF(ts) to complement a mutant pool with a gene *in trans*, and then cure the complementing plasmid to restore the original mutant phenotype. Finally, we constructed a series of cassettes that facilitate the manipulation of *oriT*, SF, and Tc^r. To construct these cassettes, each gene of interest was cloned into a mirrored cloning site so that it is flanked by *Sac*I-*Kpn*I-*Sma*I-*Bam*HI-*Xba*I-*Sal*I-*Pst*I-*Sph*I-*Hin*dIII. Thus, the cassettes can be isolated and cloned into many different restriction sites. The *oriT* cassette carried the minimum *oriT* sequence (approximately 260 bp) of RP4. The SF cassette carried 1.3 kb of minimum SF sequence. Both *oriT* and SF cassettes can be isolated with any of the restriction enzymes listed above. The Tc^r marker of pBR322 can be isolated with *Kpn*I, *Sma*I, *Xba*I, or *Pst*I restriction enzymes. The tools described in this report have been used with success in our laboratory and allowed some unique and heretofore untried manipulations with *P. aeruginosa* that facilitated studies on the molecular mechanisms of its pathogenesis.

D-150. Isolation and Characterization of a Temperature-sensitive (ts) FabG (β-Ketoacyl-ACP Reductase) Mutant from *Pseudomonas aeruginosa*

J. Huang, X. Jiang, S. Pearson, C. Traini, D. McDevitt
SmithKline Beecham Pharmaceuticals, Collegeville, PA

We have used chemical (ethyl methanesulfonate) mutagenesis to isolate temperature-sensitive (ts) mutants in an attempt to identify essential *P. aeruginosa* gene products to serve as antibiotic targets. Over 150 mutants, which show ts growth on complex medium at 44°C, have been isolated. A genomic library containing 5 to 6 kb DNA fragments of wild-type *P. aeruginosa* was constructed to complement these ts mutants. One of the ts mutants was complemented by clone pTS67 *in trans*, which contains a 5.4 kb DNA fragment harboring the *plsX-fabD-fabG-acpP-fabF* gene cluster. Deletion/subcloning analysis showed that a subclone containing only *fabG* was able to complement the ts mutant indicating that the mutation(s) responsible for the ts phenotype was present in the *fabG* gene. Amplification of the *fabG* gene by PCR and comparison of sequences from the wild type and the mutant revealed a single missense mutation (C to T) in the *fabG* gene from the ts mutant. The mutation causes an amino acid substitution resulting in a change of a conserved Arg residue at position 135 to Cys in the FabG ORF. Furthermore, a revertant (i.e., restore growth at 44°C) of the ts *fabG* mutant was isolated and found to have the original mutation (C to T) reverted back to wild type sequence (T to C). A second site mutation within the ts *fabG*, resulting in Leu80 to Phe, also restored growth at the non-permissive temperature. Flow cytometry studies showed that the growth of the ts *fabG* mutant at the non-permissive temperature was severely inhibited but the cells were still viable since the membrane potential and integrity were not compromised. Microscopy studies revealed that these cells formed chains consisting of 8-12 bacteria. In contrast, the wild type parent strain and the revertant of the ts *fabG* mutant grew normally at the non-permissive temperature and did not form chains of cells.

D-151. In Search of RpoS Regulated Genes in *Pseudomonas aeruginosa*

S. J. Suh, L. A. Silo-Suh, D. E. Ohman
Virginia Commonwealth University and
McGuire VA Medical Center, Richmond, VA

The sigma factor RpoS plays diverse roles in the physiology of the opportunistic bacterial pathogen *Pseudomonas aeruginosa*. As in other bacteria, RpoS mediates the general stress response of *P. aeruginosa* against heat shock, osmotic stress, and oxidative stress. However, the RpoS of *P. aeruginosa* is also required for maximum production of virulence factors like exotoxin A, elastase, and alginate. Likewise, RpoS negatively affects maximum production of pyoverdine and pyocyanin. Interestingly, RpoS does not appear to be involved in protection against prolonged carbon starvation in *P. aeruginosa*. To explore the activities of RpoS in *P. aeruginosa*, we undertook a comprehensive search of genes that are under its regulation. In a proteome analysis evaluating total cellular proteins from a wild-type strain (PAO1) and its *rpoS101::aacCI* (SS24) mutant, we determined that RpoS affects the accumulation of at least 25 proteins in *P. aeruginosa*. At least fifteen proteins, designated Sip (for RpoS induced proteins) required RpoS for maximum accumulation, and at least ten proteins, designated Srp (for RpoS repressed proteins) were decreased in the presence of RpoS. Four Sips and one Srp were chosen for amino terminal sequencing to determine the identities of the proteins. Based on our analysis, Sip12 is a chaperone regulated by the heat-shock sigma factor in *E. coli*. Sip17 shares homology with the elongation factor Tu of *Escherichia coli*. Sip18 shares homology with a general stress protein of *Thermotoga maritima* and *Bacillus subtilis*. Sip19 is a protein of unknown function that may be regulated by a *lysR* family transcriptional regulator. Srp4 shares homology with an *E. coli* octomeric hydrolase. The promoter regions for the genes that encode these proteins were cloned in a *lac* fusion vector, and the effects of RpoS on their expression in *P. aeruginosa* were assayed. We also took a genetic approach to identify other RpoS regulated genes in *P. aeruginosa*. We isolated 35,000 insertions of a promoter probing transposon, mini-Tn*5* B21, in the genome of a *rpoS* mutant, and then pooled the insertion mutants according to the *lac* reporter gene phenotype. We are currently screening the Tn insertion pools to identify other RpoS regulated genes in *P. aeruginosa*.

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426.2

Negative Cooperativity of Substrate Binding in Human Glutathione Synthetase

Jia-Li Luo, Mary E Anderson. Microbiology and Molecular Cell Sciences, The University of Memphis, Life Science Building, Memphis, TN 38152

Human glutathione synthetase (GS) catalyzes the last step in glutathione (GSH) biosynthesis. It is a homodimer with a monomer subunit MW of 52 kDa. In this study, purified recombinant wild type human GS was subjected to analysis by Steady-state kinetics. Kinetic analysis reveals a departure from linearity of the Lineweaver-Burk double reciprocal plot for the binding of γ-glutamyl substrate, indicating cooperative binding. The measured apparent Km values for γ-glutamyl-α-aminobutyrate (an analog of γ-glutamylcysteine) are 63 and 164 μM, respectively. Neither ATP (Km, 248μM), nor glycine (Km, 452μM) exhibits such cooperative binding behavior. Although ATP is proposed to play a key role in the sequential binding of γ-glutamyl substrate to the enzyme, the cooperative binding of the γ-glutamyl substrate is not affected by alterations of ATP concentration. Quantitative analysis of the kinetic results for γ-glutamyl substrate binding gives a Hill coefficient (h) of 0.75, indicating negative cooperativity. Our studies, for the first time, show that human GS is an allosteric enzyme with cooperative binding for γ-glutamyl substrate.

426.3

Kinetic Mechanism of beta-ketoacyl-ACP Reductase (FabG) with an Alternate Substrate

Michael R. Dermyer[1], Karen E. Siegel[2], Michael J. Melnick[3], Tod P. Holler[1]. [1]Antibacterials Molecular Sciences, Pfizer Global Research and Development, 2800 Plymouth Rd., Ann Arbor, Michigan 48105, [2]Antibacterials Pharmacology, Pfizer Global Research and Development, Ann Arbor, Michigan, [3]Antibacterial Medicinal Chemistry, Pfizer Global Research and Development, Ann Arbor, Michigan

The search for new pharmaceuticals to address the growing problem of drug-resistant bacteria has led us to study enzymes in the bacterial fatty acid biosynthesis pathway, including beta-ketoacyl-ACP reductase (FabG), the product of the *fabG* gene. The natural substrate for this enzyme, beta-ketoacyl-acyl carrier protein, is difficult obtain in quantities sufficient for drug discovery efforts, so we have chosen to study more readily available substrates for this enzyme. We have found that beta -hydroxybutyryl CoA is a suitable substrate for assaying FabG in the reverse of its physiological direction. Under these conditions, we have determined that FabG follows an ordered Bi Bi kinetic mechanism, with $NADP^+$ binding first. We also find that both substrates, beta -hydroxybutyryl CoA and $NADP^+$, exhibit competitive substrate inhibition.

426.4

Salt Effects on β-Glucosidase Kinetics

Lindsey O Ragland, Larry D Byers. Department of Chemistry, Tulane University, 1027 Stern Hall 6400 Freret Street, New Orleans, LA 70118

The effects of various salts on the equilibria for substrate and product binding, as well as on the steady-state kinetics, of sweet almond β-glucosidase (EC 3.2.1.21) were investigated. Salts, such as NaCl, were found to be inhibitory over a wide pH range. The kcat for p-nitrophenyl glucoside (p-NPG) is reduced in the presence of added salt, but Km remains essentially unchanged. This suggests that Km= Ks, the thermodynamic dissociation constant of the ES complex. This is also consistent with the fact that

kcat depends on pH and temperature while Km does not. Since the reaction mechanism is known to involve a glucosyl-enzyme (acylal) intermediate, the salt effect on the kinetic parameters requires that hydrolysis of this intermediate must be faster than its formation. This is consistent with the absence of a pre-steady-state burst of p-nitrophenol release. The second-order rate-constant for formation of the covalent intermediate (107 M-1min-1 at pH=5.7, m=0.1 M) has an enthalpy of activation of 6.8 kcal/mol, which is over 22 kcal/mol more favorable than that for the spontaneous hydrolysis of p-NPG.

426.5

Characterization of the kinetic properties and subcellular distributions of the AMPD2 (isoform L) spliceoforms

Amy Louise Haas, Richard L Sabina. Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

AMP Deaminase (AMPD) is a highly regulated enzyme that catalyzes the hydrolytic deamination of AMP to IMP. Three isozymes of AMP deaminase, AMPD1, AMPD2, AMPD3, are produced by a multigene family in human tissues and cells. Each isozyme exhibits sequence variation at its distal N-terminal end. Of the three isozymes, the least is known about the three variants of isoform L, which differ by N-terminal extensions of 47 (AMPD2-1A/2), 128 (AMPD2-1B/2), or 53 amino acids (AMPD2-1B/3). We have expressed these proteins to determine whether the N-terminal extensions affect their kinetic parameters or subcellular localizations. Phosphocellulose chromatography was used to purify each variant and similar kinetic properties were observed ($p>0.05$ for all comparisons in two-tailed t-tests). AMPD2-1A/2, -1B/2, and -1B/3 show Kmapps of 8.7±0.8 mM, 14.0±5.0 mM, and 11.4±4.3 mM, respectively (n=3 for each). Addition of ATP lowers the Kms to 1.1±0.3 mM, 1.0±0.2 mM, and 1.0±0.4 mM, respectively. The -1A/2 variant differs from -1B/2 and -1B/3 in its allosterism in the absence of ATP, with a Hill coefficient of 2.3±0.5, as compared to 1.2±0.3 (-1B/2; $p<0.01$), and 1.5±0.4 (-1B/3; $p<0.05$). When expressed as GFP fusion proteins in HeLa cells, all three AMPD2 variants are cytoplasmic. These combined data demonstrate that N-terminal extensions to the AMPD2 polypeptide do not significantly affect its kinetic parameters or subcellular distributions, but may alter its allosteric cooperativity.

426.6

Determination of Kinetic Constants K_M and k_{cat} for K73R β-Lactamase

Jennifer L. T. Keeling, Mark Hokenson, Anthony L. Fink. Chemistry, University of California, Santa Cruz, 1156 High St., Santa Cruz, CA 95064

β-Lactamases are the primary means of defense for pathogenic bacteria against penicillin and cephorosporin type antibiotic medications. These hydrolytic enzymes catalyze the acylation and opening of the β-lactam amide ring to produce inactive antibiotics. The exact mechanism by which this occurs remains controversial and uncertain. Lysine 73 is a conserved active site residue in the class A β-lactamases, as well as other members of the serine-penicillin sensitive enzyme family. Its role in catalysis is believed to play a part in acylation. To determine Lysine 73's function, mutation of Lysine 73 to Arginine,K73R β-lactamase, has been studied. Kinetic analysis of K73R against known substrates of wt β-lactamase was done by using UV spectrophotometry at varying pHs. The kinetic constants, K_m and k_{cat}, were determined for a variety of penicillin and cephorosporin substrates. These results provide information about the role Lysine 73 plays in acylation and deacylation in β-lactamase catalysis, and brings us closer to understanding and elucidating the complete mechanism by which β-lactamase hydrolyzes β-lactam amide rings. This information is necessary for the design and synthesis of antibiotics that are resistant to β-lactamase.

426.7

Cholesterol/Oxysterol Sulfotransferase (SULT2B1): Functional and Structural Characterization.

Hirotoshi Fuda, Young C. Lee, Chikara Shimizu, Norman B. Javitt, Charles A. Strott. NICHD/ERRB, National Institutes of Health, 9000 Rockvillepike, Bldg.49, Rm.6A36, Bethesda, MD 20892.

The biotransformation of cholesterol and oxysterols by sulfonation is a fundamental process. This reaction is carried out by two isoforms of a unique hydroxysteroid sulfotransferase. We have determined the kinetic parameters and key structural elements necessary for catalytic activity of the isoforms. Whereas the K_ms for SULT2B1a and SULT2B1b are similar, i.e. 0.7 and 0.5μM, respectively, V_{max}s are strikingly different. The V_{max} for SULT2B1b is an order of magnitude higher than that for SULT2B1a, i.e. 0.65 and 0.06 nmol/mg/min, respectively. The SULT2B1a/b isoforms are distinct from all other cloned cytosolic sulfotransferases in that they have extended amino and carboxy-terminal ends, the functional significance of which is not appreciated. Otherwise, the core of the SULT2B1a/b proteins contains structural features that are highly conserved among the cytosolic sulfotransferases. Removal of the carboxy-terminal end has no effect on catalytic activity, whereas removal of the shorter amino-terminal extension results in a complete loss of catalytic activity. Further mutational analyses identified a four amino acid sequence near the amino terminus that is required for full catalytic activity.

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Substrate and Potassium Effects on Glycine N-Methyltransferase of Halophilic Methanoarchaea

M. Lai, C. Wang, Y. Wu

Methanohalophilus portucalensis FDF1 can de novo synthesize betaine, through the methylation of glycine, as osmolyte to encounter the osmotic stress. The activity of glycine N-methyltransferase (GNMT) which formed sarcosine by transfer the methyl group from S-adenosylmethionine (AdoMet) to glycine was detected by radiometric methods in extracts of M. portucalensis FDF1. GNMT was further purified by DEAE-Sephacel ion-exchange chromatograph with the step potassium gradient (0.1-0.5 M). The estimated molecular weight of GNMT was 303 kDa and was composed by three non-identical subunits with molecular weight within the range of 50-55 kDa. In addition to transfer the methyl group from AdoMet to glycine, GNMT also showed the enzyme activities of transferring the methyl group to sarcosine and dimethylglycine with specific activity of 0.39 and 0.43 nmole/ug·hr protein, respectively. The increasing level of potassium enhanced the methyl transfer activity. Results indicated that GNMT of halophilic Methanoarchaea is a potassium regulated, broad substrate spectrum methyltransferase.

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Nucleic Acids Research, 1996, Vol. 24, No. 9 1747–1752

Identification of promoter and stringent regulation of transcription of the *fabH*, *fabD* and *fabG* genes encoding fatty acid biosynthetic enzymes of *Escherichia coli*

Sergey M. Podkovyrov and Timothy J. Larson*

Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0308, USA

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ABSTRACT

In *Escherichia coli*, amino acid starvation results in the coordinate inhibition of a variety of metabolic activities, including fatty acid and phospholipid biosynthesis. By using primer extension analysis we identified the *fabH* promoter responsible for transcription of the *fabH*, *fabD* and *fabG* genes encoding fatty acid biosynthetic enzymes. The response of the *fabH* promoter to amino acid starvation was determined *in vivo*. Transcripts originating from the *fabH* promoter were quantified by employing a ribonuclease protection assay. The *fabH* promoter was subject to *relA*-dependent stringent control and was repressed ~4-fold upon amino acid starvation. The results suggest that inhibition of transcription initiation of lipid biosynthetic genes in starved cells contributes to the stringent control of lipid biosynthesis.

INTRODUCTION

Escherichia coli and other bacteria have effective adaptation mechanisms that help them survive unfavorable environmental conditions such as nutritional stresses or temperature shifts. An example of rapid adaptation is the cellular response to amino acid starvation, termed the stringent response. In *E.coli*, amino acid deprivation results in the coordinate inhibition of a variety of metabolic activities, including stable RNA synthesis, protein synthesis and lipid synthesis (see 1 for review). Amino acid deficiency results in binding of codon-specified uncharged tRNA to ribosomes which activates the ppGpp synthetic activity of the ribosomally bound RelA protein. Accumulation of ppGpp leads to a highly specific inhibition of the transcription of stable RNA genes (2). In a relaxed *relA* strain ppGpp levels fail to increase with the onset of amino acid starvation and stable RNA synthesis continues (3). ppGpp has been proposed to modify RNA polymerase, thereby altering the pattern of transcription initiation from stable RNA promoters (4). Recently, direct interaction of ppGpp with *E.coli* RNA polymerase has been demonstrated (5).

One of the pleiotropic effects of the stringent response is an immediate inhibition of fatty acid and phospholipid biosynthesis which occurs in *relA*$^+$ but not in *relA* strains (6). Induction of expression of an unregulated, truncated *relA* gene situated on a multicopy plasmid leads to elevated ppGpp levels and inhibition of *de novo* fatty acid and phospholipid synthesis (7). These data suggest that ppGpp is involved in the inhibition of fatty acid and phospholipid synthesis, but little is known about the mechanisms of inhibition. There are several reports that ppGpp can inhibit *in vitro* some enzymes participating in synthesis of fatty acids and phospholipids (8,9). Rock and co-authors demonstrated that *sn*-glycerol-3-phosphate acyltransferase is inhibited upon induction of ppGpp synthesis *in vivo* (7). Their data pointed to a direct biochemical interaction between the enzyme and ppGpp. To our knowledge, all studies reported to date concerning the mechanisms of inhibition of fatty acid and phospholipid synthesis dealt with effects of ppGpp on the biosynthetic enzymes. In this paper, for the first time, we present data regarding regulation of transcription of lipid biosynthetic genes during amino acid starvation.

Recently we demonstrated that the *g30k* gene of unknown function, the *rpmF* gene encoding ribosomal protein L32, the *plsX* gene encoding a protein involved in membrane lipid synthesis and the *fabH*, *fabD* and *fabG* genes encoding several fatty acid biosynthetic enzymes comprise an operon (10; Fig. 1). We found that, in addition to the operon promoters located upstream of *rpmF*, there is an internal promoter located within the *plsX* gene. The goal of the present study was to identify this promoter and to test if it is subject to stringent control.

MATERIALS AND METHODS

Strains and growth media

All bacterial strains used in this study were *E.coli* K-12 derivatives. DH5αF′[ϕ80*d lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*] (Gibco BRL, Gaithersburg, MD) was used as the host for DNA manipulations. TL504[Δ(*lac-ZYA-argF*)*U169 zah-735*::Tn*10*] was derived from wild type strain MG1655(11) by P1 transduction with strain SH205 (12) as donor,

* To whom correspondence should be addressed

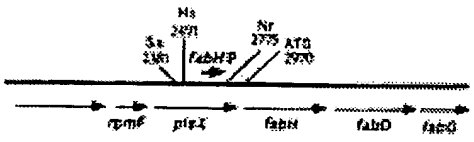

Figure 1. Structure of the *rpmF-plsX-fab* operon. *fabH* P indicates the *fabH* promoter identified in this study. Restriction sites are abbreviated as follows: Ss, *Ssp*I; Hi, *Hinc*II; Nr, *Nru*I. ATG, the start codon of the *fabH* gene. Numbering of nucleotides starts from the first base of the *Pst*I site located within the *orfX* gene upstream of *g30k* (42). Figure is not drawn to scale.

with selection for tetracycline resistance. This *relA*$^+$ Δ*lac* strain was used for isolation of RNA. The *fabH* promoter was assayed in parallel in both MC4100[*relA1* Δ*lac*] (13) and XZ132 [MC4100*relA*$^+$] (14). For experiments to test stringent control, A and B salts of Clark and Maaløe (15) were supplemented with 0.4% glucose, uracil (50 μg/ml), thiamine (10 μg/ml), and all 20 amino acids except serine (each at 40 μg/ml). Cells were grown at 37°C to an A_{600} of 0.5 and amino acid starvation was induced by addition of serine hydroxamate to 400 μg/ml. In all other experiments Luria–Bertani medium (16) was used. When needed, media were supplemented with 100 μg/ml of ampicillin.

Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems model 381A DNA synthesizer and purified using oligonucleotide purification cartridges (Cruachem, Dulles, VA) as recommended by the manufacturer. The following oligonucleotides, with their 3′ coordinates and references for the DNA sequences, were used in this study:

1. 5′-TGGCGGCTGTGGGATTAACTGCG-3′ (6679) (17)
2. 5′-GCGAGAATTCAAGATGCTGAAGATCAG-3′ (4044) (18)
3. 5′-GCTAGGATCCGTCATGCCATCCGTAAG-3′ (3824) (18)
4. 5′-GCGAGAATTCTATGACCATGATTACGG-3′ (13) (19)
5. 5′-GATCGATCCCATTCAGGCTGCGCAAC-3′ (150) (19)
6. 5′-AATTCCTCTTGTCAGGCCGGAATAACTCC-CTATAATGCGCCACCACTG-3′ (1229) (20)
7. 5′-GATCCAGTGGTGGCGCATTATAGGGAGTT-ATTCCGGCCTGACAAGAGG-3′ (1186) (20)

Construction of plasmids and DNA manipulations

Plasmids used in this study are listed in Table 1. Plasmids pSPlac and pSPbla were constructed by cloning appropriate PCR fragments into the *Eco*RI and *Bam*HI sites of pBluescript KS(+) (Stratagene, La Jolla, CA). pSP417 was the template for PCR; primers 2 and 3 were used for amplification of the *bla* fragment and primers 4 and 5 were used for amplification of the *lac* fragment. T3 and T7 primers were used to sequence the fragment inserts of pSPlac and pSPbla.

For plasmid DNA purification, Wizard Minipreps DNA Purification System was employed (Promega, Madison, WI). DNA fragments for cloning were isolated from agarose gel by using Wizard PCR Preps DNA Purification System (Promega). Insertions of recombinant plasmids were sequenced by the chain termination method (21) with the Sequenase version 2.0 (Amersham, Arlington Heights, IL). PCR was performed in a standard reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl) including 3 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 1 μM of each primer, 0.5 μg plasmid DNA and 2 U *Taq* polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, CT) at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s in a total of 35 cycles. All other standard molecular biology techniques were based on methods described elsewhere (16).

Purification of RNA and primer extension analysis

Total RNA was isolated by using guanidine isothiocyanate for cell lysis and rapid inactivation of cellular RNases (22) (all reagents for isolation of RNA were purchased from 5 Prime→3 Prime, Inc., Boulder, CO). The quality of RNA was determined by visualization of distinct ribosomal RNA bands on a denaturing formaldehyde gel (16).

For primer extension analysis, primer 1 was 5′ end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled primer, 1 pmol, was mixed with 5 μg total RNA in a final volume of 8 μl. The mixture was boiled for 2 min and cooled on ice. The hybridized primer was extended by addition of all four dNTPs at 0.7 mM each, reverse transcriptase buffer and 50 U Moloney murine leukemia virus reverse transcriptase (both purchased from New England Biolabs, Beverly, MA) in a total volume of 15 μl, followed by incubation at 42°C for 30 min. The reaction was stopped by addition of 15 μl gel loading buffer containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol. The extension products were resolved on a 5% polyacrylamide sequencing gel.

Table 1. Plasmids used in this study

Plasmid	Relevant characteristic	Source
pSP417	vector for construction of *lacZ* transcriptional fusions	(24)
pSP413	*fabH* promoter cloned into pSP417	this study
pSP17	*rrnB* P1 promoter cloned into pSP417	this study
pSS20	*lacUV5* promoter cloned into pSP417	S. Solow (this lab.)
pBluescript KS(+)	multiple cloning site flanked by T3 and T7 promoters	Stratagene
pSPlac[a]	5′ part of *lacZ* gene cloned into pBluescript KS(+)	this study
pSPbla[a]	5′ part of *bla* gene cloned into pBluescript KS(+)	this study

[a]For coordinates of the cloned fragments see coordinates of oligonucleotides 4 and 5 (for *lac*), and 2 and 3 (for *bla*) in Materials and Methods.

In vitro transcription

To prepare probes for RNase protection assays, plasmids pSPlac and pSPbla were linearized using *Hin*dIII and transcribed *in vitro* in the presence of 0.5 mM each ATP, GTP and CTP, 50 μM UTP, 50 μCi [α-^{32}P]UTP and T3 RNA polymerase as described in Ambion technical bulletin for MAXIscript *in vitro* transcription kit (Ambion, Austin, TX). Following *in vitro* transcription, the template was destroyed by the addition of 2 U RNase-free DNase (Ambion). The unincorporated [α-^{32}P]UTP was removed by passing the reaction mixture through a G-25 spin column (Boehringer Mannheim, Indianapolis, IN), and the eluate containing the probe was kept at −70°C. To prepare molecular weight standards, *in vitro* transcription was performed with RNA Century marker template set (Ambion).

Ribonuclease protection assay and quantitation of RNA

Ribonuclease protection assays were performed by co-precipitation of ^{32}P-labeled probe (~1 × 10^4 c.p.m.) with sample RNA (~1–10 μg). Hybridization and RNase digestion were conducted by using the RPAII kit (Ambion). Protected RNA fragments were separated on an 8 M urea, 5% polyacrylamide gel and detected by autoradiography. RNA was quantified by counting the radioactivity in the corresponding bands and by scanning the X-ray film using a Shimadzu CS-9000 scanning densitometer.

Assay of β-galactosidase

Enzyme activity was determined in triplicate by using logarithmically growing cells permeabilizied with sodium dodecyl sulfate and chloroform as described (23). One unit of enzyme activity was defined as described by Miller (23).

RESULTS

Identification of the *fabH* promoter

Recently we constructed a series of transcriptional fusions between different parts of the *rpmF-plsX-fab* operon and *lacZ*, and employed insertional mutagenesis to study the transcriptional organization of the operon (10). One of our conclusions was that there is an internal promoter (termed the *fabH* promoter) within the *plsX* gene responsible for ~40% of all transcripts for *fabH*, *fabD* and *fabG*. It follows from comparison of β-galactosidase activities in the cells carrying transcriptional fusions between different parts of the operon and *lacZ* that the *fabH* promoter is located between the *Ssp*I and the *Nru*I restriction sites (10; Fig. 1). To further localize the position of the *fabH* promoter we used the *Hin*cII restriction site conveniently located between *Ssp*I and *Nru*I and cloned the *Ssp*I–*Hin*cII and the *Hin*cII–*Nru*I DNA fragments upstream of the promoterless *lacZ* gene of the plasmid vector pSP417 (24; the recombinant plasmids were named pSP411 and pSP413, respectively). Expression of *lacZ* from plasmid pSP413 was much higher than that from pSP411 (18 500 U versus 1300 U) and comparable with that from the plasmids carrying the *Ssp*I–*Nru*I fragment (19 000 U; 10), which localizes the *fabH* promoter within the 283 bp *Hin*cII–*Nru*I fragment (plasmid pSP413).

To map the *fabH* promoter more precisely we performed primer extension analysis. Total RNA was isolated from exponentially growing TL504(pSP413) cells and hybridized with primer 1 (see Materials and Methods). Primer extension products were run on the same gel with products of sequencing reaction of pSP413 with the same primer. One extension product was seen (Fig. 2) suggesting that there is only one promoter located within the *Hin*cII–*Nru*I fragment of pSP413. From the nucleotide sequence of the *plsX* gene (GenBank accession number M96793) and the results of primer extension analysis (Fig. 2), we concluded that the *fabH* promoter sequence is:

5′-CCCGACAGTATAACGGCGCCTGTCTGTTAGGATTGCGCGG-3′

where the last G is the transcription start point and the −10 sequence is underlined. The transcription start site is 242 nucleotides upstream of the *fabH* translation initiation codon. There is no typical −35 region in the *fabH* promoter sequence. There is, however, a GC-rich sequence motif between the −10 region and the start site. This GC-rich motif has been called a discriminator sequence and has been shown to be a characteristic feature of all known stringently regulated promoters (25). Therefore, we decided to determine if the *fabH* promoter is subject to stringent control.

Figure 2. Mapping of the *fabH* promoter. The primer extension reaction was performed using total RNA from strain TL504(pSP413) and primer 1 (see Materials and Methods) (lane 1). The sequence ladder was generated by using the same primer and plasmid pSP413 as template. The coordinate of the transcription start point is 2727. For numbering see the legend to Figure 1.

Stringent regulation of the *fabH* promoter

Three recombinant plasmids, pSP413, pSP17 and pSS20, were used in the experiments to test stringent control of the *fabH* promoter. They all were derived from the same vector pSP417 (24), and carry the ampicillin resistant gene (*bla*) and different promoters cloned upstream of the *lacZ* gene. Plasmid pSP413 contains the *fabH* promoter. Plasmid pSP17 contains the P1 promoter of the *rrnB* ribosomal operon, a classical example of a stringently regulated promoter (26). We chose to clone the *rrnB* P1 core promoter (−42; +4) since it is 300-fold less active than the wild-type promoter with the upstream sequences, but is inhibited by amino acid starvation to the same extent as the full-length promoter (27). P1 was assembled from the oligonucleotides 6 and 7 and cloned into the *Eco*RI and *Bam*HI sites of pSP417. Plasmid

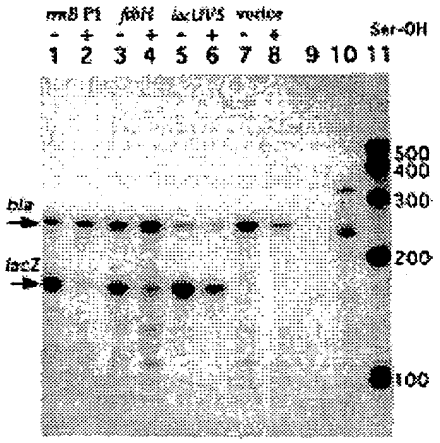

Figure 3. Stringent response of the *fabH* promoter. RNase protection assays were performed on total RNA isolated from strain TL504 containing plasmids pSP17 (lanes 1 and 2), pSP413 (lanes 3 and 4), pSS20 (lanes 5 and 6) or pSP417 (lanes 7 and 8). RNA was hybridized with a mixture of two radiolabeled probes complementary to the *lacZ* and the *bla* mRNA. Lanes 9 and 10, probes were hybridized with yeast RNA. Samples shown in lanes 1–9 were digested with RNase A and RNase T1. Lane 10, non-digested probes; lane 11, RNA Century Markers (Ambion) with length of RNA (in bases) on the right. Ser-OH, serine hydroxamate treatment. Arrows indicate positions of protected *lacZ* and *bla* probes.

pSS20 contains the *lacUV5* promoter, which is not subject to stringent control (1) and serves as a negative control.

The stringent response of TL504 cells transformed with plasmids pSP413, pSP17, pSS20 and pSP417 was induced by addition of serine hydroxamate to the cultures grown in a medium lacking serine. The effect of amino acid starvation on the selected promoters was determined by comparison of *lacZ* mRNA levels in the cells that received or did not receive serine hydroxamate. To compensate for any differences in plasmid copy number or yield of RNA, the level of *bla* transcription was used as an internal control [it is known that transcription of the *bla* gene is not affected by amino acid starvation (28)]. The levels of the *lacZ* and *bla* mRNA were quantified by using ribonuclease protection assays. Plasmids pSPlac and pSPbla were used as templates to synthesize radiolabeled RNA probes complementary to the 5′-parts of the *lacZ* and *bla* mRNA, respectively. Both probes were added simultaneously to the same RNA sample, eliminating experimental variability of separate detection of multiple mRNA targets and making quantitation of the *lacZ* mRNA highly accurate and reproducible.

The results of the assays are shown in Figure 3. There is no band corresponding to the *lacZ* mRNA in the case of vector pSP417 (lanes 7 and 8), since four copies of the strong transcriptional terminator *T1* from the *E.coli rrnB* operon block transcription from upstream plasmid promoters toward the promoterless *lacZ* gene (29). The non-stringent *lacUV5* promoter did not change its activity upon amino acid starvation (the *lacZ/bla* ratio was constant, lanes 5 and 6), while transcription from *rrnB* P1 was significantly reduced (lanes 1 and 2). It can be seen that the *fabH* promoter also showed stringent repression (lanes 3 and 4). The results of the *lacZ* mRNA quantitation (*lacZ/bla* mRNA ratios) are shown in Figure 4. Transcription from the *fabH* promoter is repressed ~4-fold after amino acid starvation. Transcription from *lacUV5* is unchanged;

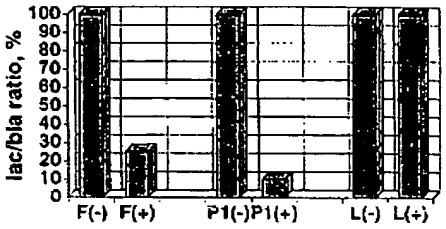

Figure 4. Quantitation of stringent control for different promoters. F, the *fabH* promoter; P1, the *rrnB* P1 promoter; L, the *lacUV5* promoter. (+), *lacZ/bla* mRNA ratio was determined 30 min after induction of amino acid starvation. (–), starvation was not induced. The value for unstarved cells was set to 100% in each case. Quantitation of RNA was reproducible with an error range of ±15% and represents the averages of at least three independent experiments.

transcription from *rrnB* P1 decreased ~10-fold, and agrees with previously published data (27).

In order to determine effect of the *relA* allele on transcription from the *fabH* promoter we used cogenic *relA1* and *relA*$^+$ bacterial strains transformed with pSP413. The level of transcription from the *fabH* promoter in starved and unstarved cells of each strain was determined by ribonuclease protection assays as described above. As seen in Figure 5, the *lacZ/bla* mRNA ratio (which is a corrected measure of the level of transcripion from the *fabH* promoter) is reduced after onset of starvation in the stringent strain XZ132 (lanes 3 and 4). The absence of this effect in the relaxed strain MC4100 (lanes 1 and 2) shows that the starvation response of the *fabH* promoter is dependent on the wild-type *relA* gene.

DISCUSSION

In our previous work we have shown that the *fabH, fabD* and *fabG* genes are part of the *rpmF-plsX-fab* operon (10). In the present study we mapped an internal *fabH* promoter of the operon located within the *plsX* gene. The three genes transcribed from the *fabH* promoter are *fabH, fabD* and *fabG*. *fabH* encodes 3-ketoacyl-ACP synthase III, the enzyme that catalyzes the first condensation reaction of fatty acid biosynthesis (see 30 for a review of fatty acid biosynthesis). Malonyl-ACP required for this step is produced by the action of malonyl CoA-ACP transacylase (encoded by *fabD*). Mutants deficient in malonyl CoA-ACP transacylase require both saturated and unsaturated fatty acids for growth (31). *fabG* encodes 3-ketoacyl-ACP reductase, the first enzyme participating in each cycle of chain elongation. The FabD, FabH and FabG proteins catalyze the successive reactions and organization of their genes into an operon is likely to be a means for coordinate regulation. In *E.coli*, genes are often organized in operons for coordinate control of transcription from the operon promoter. Some operons, however, have more complex regulatory mechanisms such as transcription from multiple promoters (32), intra-operon attenuation (33) or differential decay of the polycistronic mRNA (34). Internal promoters have been discovered in a number of operons including an operon containing genes for ribosomal protein, DNA primase and σ factor of RNA polymerase (sigma operon; 35) and an operon containing genes for ribosomal proteins and β and β′ subunits of RNA polymerase (beta operon; 33). The presence of promoters internal to the operon makes regulation of gene expression more flexible permitting coordinate expression in some situations and discoordinate expression in others. For example, regulation of both

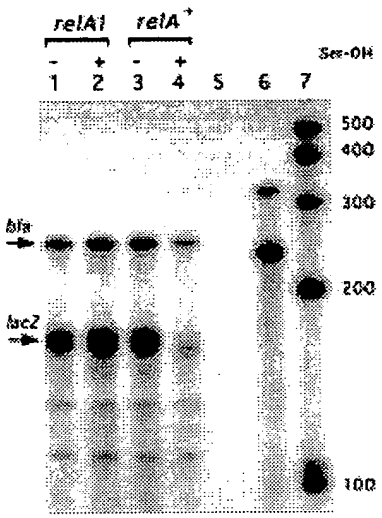

Figure 5. Transcription from the *fabH* promoter in *relA*$^+$ and *relA1* strains after amino acids starvation. Total RNA was extracted from MC4100(*relA1*) (lanes 1 and 2) and XZ132(*relA*$^+$) (lanes 3 and 4) cells and hybridized with RNA probes specific to the *lac* and *bla* mRNA. Lanes 5 and 6, probes were hybridized with yeast RNA. Lanes 1–5, hybridized probes were digested with RNase A and RNase T1; lane 6, non-digested probes; lane 7, RNA Century Markers (Ambion) with length of RNA (in bases) on the right. Ser-OH, serine hydroxamate treatment. Arrows indicate positions of protected *lacZ* and *bla* probes. For an unknown reason the radiolabeled RNA probe complementary to *lacZ* generated two nearly identical bands of ~170 bases. These bands correspond to the transcript originating from *fabH* and were absent when only the *bla* probe was used or when both probes were hybridized with total RNA isolated either from MC4100(pSP417) or XZ132(pSP417) (data not shown).

RNA polymerase and ribosomes is relatively coordinate upon changes in growth conditions (36), but in the case of heat shock, the presence of an internal promoter in the sigma operon allows discoordinate regulation by selective activation of transcription of the *rpoD* gene encoding sigma factor (35,37). We suggest that the *fabH* promoter and the operon promoters located upstream of the *rpmF* gene may respond differently to some environmental signals, but at present these signals are not identified.

The striking feature of the *fabH* promoter found in this work is its stringent regulation. By and large, studies on stringently regulated promoters are limited to genes that encode products involved in ribosome function. Recently stringent control has been demonstrated for the *dnaA* (38) and *fis* (39) genes encoding the DnaA protein involved in DNA replication and the Fis protein involved in a number of cellular processes including the transcriptional activation of stable RNA synthesis. Our results show that transcription of genes encoding fatty acid biosynthetic enzymes is also subject to stringent control. Thus, the stringent control of transcription may be a mechanism for inhibition of some anabolic cellular functions during amino acid starvation.

One of the numerous effects of amino acid starvation on cellular physiology and metabolism is a *relA*-dependent inhibition of fatty acid and phospholipid synthesis (6). A target for stringent control of lipid synthesis has not been defined precisely, however. Rock and co-workers showed that overexpression of the *plsB* gene encoding *sn*-glycerol-3-phosphate acyltransferase relieves the inhibition of fatty acid and phospholipid synthesis induced by accumulation of ppGpp (7). It should be noted that in their work ppGpp accumulation was achieved by induction of expression of the *relA* gene located on a plasmid, and, in contrast to induction by amino acid starvation, phospholipid biosynthesis was not completely abolished in induced cells. Thus, cell responses to amino acid starvation and *relA* overexpression are different and a target for stringent control of lipid synthesis in these two cases may be different. The *sn*-glycerol-3-phosphate acyltransferase catalyzes the first step in phospholipid biosynthesis by condensation of *sn*-glycerol-3-phosphate and fatty acylthioesters to yield lysophosphatidic acid. Since the preceding step, formation of fatty acids, requires >90% of the ATP consumed in lipid biogenesis, it appears to be likely that an early step in fatty acid biosynthesis could be a primary site for stringent regulation. Our data indicate that the *fabH* promoter is subject to stringent control. This means that transcription of fatty acid biosynthetic genes (*fabH*, *fabG* and *fabD*) is inhibited upon amino acid starvation. Especially noteworthy in this regard is that the FabH protein is thought to be a regulator of fatty acid biosynthesis in bacteria (40,41). Our findings show that one potential mechanism of inhibition of fatty acid biosynthesis upon amino acid starvation may be realized through ppGpp-dependent inhibition of transcription of the pathway genes. On the other hand, an immediate effect of ppGpp inhibition may be caused by direct biochemical interaction between ppGpp and the corresponding biosynthetic enzyme(s). We suggest that due to the complexity of the changes taking place during the stringent response, inhibition of fatty acid and phospholipid biosynthesis is a complex event with controls exerted at both the transcriptional and enzymatic levels. Also, some inhibitory effects of ppGpp may be indirect or part of a regulatory cascade.

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The Gene Encoding *Escherichia coli* Acyl Carrier Protein Lies within a Cluster of Fatty Acid Biosynthetic Genes*

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Merriann Rawlings‡ and John E. Cronan, Jr.

From the Departments of Microbiology and Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

The gene encoding *Escherichia coli* acyl carrier protein (ACP) has been isolated and sequenced. The ACP gene (called *acpP*) was located on the genetic map between *fabF* and *fabD* which encode two fatty acid biosynthetic enzymes, 3-ketoacyl-ACP synthase II and malonyl CoA-ACP transacylase, respectively. An open reading frame between *acpP* and *fabD* encodes a 26.5-kDa protein that has significant sequence identity (>40%) with two acetoacetyl-CoA reductases and thus is believed to encode a 3-ketoacyl-ACP reductase. This gene (called *fabG*) is cotranscribed with *acpP*. Thus, the gene encoding ACP, the key carrier protein of fatty acid synthesis, is located within a cluster of fatty acid biosynthetic genes.

Acyl carrier protein (ACP)[1] plays a key role in lipid biosynthesis in bacteria (1) and plants (2). ACP carries the nascent fatty acid chain esterified to the thiol group of the 4′-phosphopantetheine prosthetic group and delivers the finished acyl chain to the acyltransferases catalyzing complex lipid synthesis (phospholipids and lipid A) (1, 2). Acyl-ACP has also been reported as an acyl donor in protein acylation (3). *Escherichia coli* ACP and its acyl forms thus interact with at least 12 different *E. coli* enzymes. The ACPs of other bacteria and plants are very similar to that of *E. coli*; all are small (<90 residues) acidic proteins modified with 4-phosphopantetheine with strong similarities of the sequences neighboring the modification site (1, 2). Indeed, several of these proteins are known to function with various of the ACP-dependent enzymes of *E. coli in vitro* (2) and *in vivo* (2, 4) suggesting similar solution structures. The large polyfunctional proteins that catalyze fatty acid synthesis in mammals (5) and fungi (6) contain 4′-phosphopantetheine-modified domains with strong sequence similarity to *E. coli* ACP. ACP-like proteins also function as acyl group carriers in the biosynthesis of polyketide (7) and polyamino acid antibiotics (8).

ACP has also been shown to function in three unexpected areas of metabolism: (i) as a cofactor in the synthesis of the membrane-derived oligosaccharides found in the periplasm of *E. coli* (9); (ii) as an essential component in the induction of nitrogen-fixing nodules by *Rhizobia* (where ACP appears involved in the synthesis of acylated oligosaccharides (10); and (iii) most recently as a subunit of mitochondrial NADH-ubiquinone oxidoreductases (11, 12).

E. coli ACP is the paradigm of this class of proteins. *E. coli* ACP was the first such protein isolated (13), the first in which the primary sequence was determined (14), and is the only ACP of known solution structure (15, 16). Despite the continuing interest in *E. coli* ACP, the gene encoding this protein had not been isolated, and no mutants are available. We report the isolation of the ACP-encoding gene and its location within a cluster of genes encoding known enzymes of fatty acid synthesis.

EXPERIMENTAL PROCEDURES

All bacterial strains used in this study were derivatives of *E. coli* K-12. Strains JM103 (17) and F⁻ M15A (18) have been described elsewhere. Strain DB6430 (F⁻*argE* Δ(*lac-pro*) rif^r, nal^r) was used as a source of chromosomal DNA. Strains L48 (*fabD89*), DM57 (*fabB20 zfa*::Tn*10*), and DM83 (*fabF3 fabB20*) used to map the kanamycin resistance (Kan^R) determinant were described previously (19). The growth media and genetic methods were as described (19).

Plasmid pMR23 was constructed by ligation of a 0.9-kbp *Pst*I-*Sca*I fragment from Tn*9* into pACYC177 (20) digested with the same two enzymes. Plasmid pMR24 was constructed by ligating the 2.6-kbp *Eco*RI-*Pst*I chromosomal fragment (Fig. 1) from the M13 mp18 clone containing the *acpP* region to pMR23 digested with the same enzymes. Plasmid pMR33 was constructed by ligating the 1.5-kbp *Eco*RI-*Pvu*II fragment of pMR24 into pTZ19R (21) digested with *Eco*RI and *Hin*cII. Plasmid pMR36 is a derivative of pMR24 having the 2.1-kbp *Kpn*I fragment in the opposite orientation. Plasmid pMR39 was constructed by inserting the Kan^R gene excised from plasmid pUC4K (Pharmacia LKB Biotechnology Inc.) with *Hin*cII into the *Nru*I site of pMR33 and was used to introduce the Kan^R determinant into the chromosome by homologous recombination (19). Plasmid pMR48 was derived from the 1.1-kbp *Pst*I-*Pvu*II chromosomal fragment modified to include a second flanking *Pst*I site. This *Pst*I fragment was ligated to *Pst*I-digested pTZ19R (21) such that the *fabG* gene was in the orientation opposite that of the vector *lac* gene. Plasmid pMR62 was constructed by digestion of pMR24 with *Eco*RI and *Sal*I, filling of the recessed ends by DNA polymerase I, and followed by religation.

The *acpP* gene was isolated from a library of 2–3-kbp *Sal*I-*Bgl*II fragments of strain DB6430 genomic DNA ligated into M13 mp18 RF digested with *Sal*I and *Bam*HI. Strain JM103 was transformed with the recombinant DNA and the resultant plaques transferred *in situ* to nitrocellulose membranes such that single-stranded DNA was selectively retained (22). The plaques were screened (17) with the [α-^{32}P]ATP-labeled synthetic ACP gene (18) that encodes the entire protein sequence and has 88% DNA sequence identity with the *acpP* gene.

RESULTS AND DISCUSSION

Our previous attempts to isolate the ACP gene were unsuccessful despite application of several different cloning and detection strategies. It, therefore, seemed possible that DNA segments encoding ACP were somehow toxic to *E. coli*. To investigate this possibility, we assembled a synthetic gene encoding ACP (18) and, indeed, found that high level production of ACP was lethal to *E. coli* (39).[2] In light of this finding,

* This project was supported by National Institutes of Health Grant AI 15650. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M84991.

‡ Present address: Dept. of Microbiology, University of Iowa, Iowa City, Iowa 52242.

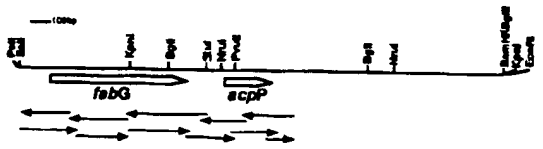
[1] The abbreviations used are: ACP, acyl carrier protein; kbp, kilobase pair; ORF, open reading frame;

[2] M. Rawlings and J. E. Cronan, Jr., manuscript in preparation.

we sought the ACP gene using the synthetic sequence as a hybridization probe and maintained cloned DNA segments in a low copy number vector to limit ACP expression.

The synthetic ACP gene proved a stringent hybridization probe in Southern blot analysis of *E. coli* chromosomal DNA fragments (data not shown). A size-selected mini-library of genomic fragments was constructed in a phage M13 vector, and recombinant phage plaques were screened with the ^{32}P-labeled synthetic gene. Screening was done under conditions allowing hybridization only to single-stranded DNA bound to the nitrocellulose filters, thus avoiding the background due to homologous sequences present in the chromosomal DNA (22). Consistent with the toxicity of the synthetic gene, the high (albeit variable) copy number of M13 clones carrying the natural gene (called *acpP*) gave spontaneously deleted variants at very high frequency, thus necessitating transfer of *acpP* to a low copy number vector to give plasmid pMR24. Even in such a plasmid, the presence of the *acpP* gene resulted in a decreased cellular growth rate.

The nucleotide sequence of the *acpP* gene region (Fig. 1) showed an open reading frame (ORF) that agreed with the ACP amino acid sequence. Note that the two published ACP amino acid sequences conflict at two positions. Vanaman *et al.* (14) reported residues 24 and 43 as Asp and Val, respectively, whereas Jackowski and Rock (23) reported residues 24 and 43 as Asn and Ile, respectively. We find residue 24 to be Asn and residue 43 to be Val. Our results are consistent with assignments from nuclear magnetic resonance analysis (15). The observed post-translational removal of the N-terminal Met is consistent with the known specificity of the aminopeptidase (24). The codon preference (25) of 1.34 (*versus* 0.48 for the randomized sequence) is consistent with this known high expression of *acpP* (about 5×10^4 molecules/cell) (1). Despite the high level of expression no sequences matching the promoter and ribosome binding sites consensus sequences are obvious.

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GTCGACACCCTGACCGCCTCGGCGCTGAACGAACCTTCAGCGATGGCAGCGGCGCTCGAGCTTTAAAAGAGGAAA   75
 V  D  T  L  T  A  S  A  L  N  E  P  S  A  M  A  A  A  L  E  L  *  fabD

ATCATGAATTTTGAAGGAAAAATCGCCACTGCTAACCGGTGCAAGCCGCCGCGAATTGGCCGCGCAATTGCTGAAACG  150
    M  N  F  E  G  R  I  A  L  V  T  G  A  S  R  G  I  G  R  A  I  A  E  T   24
 fabG

CTCCGCAGCCCGTGGCGGGAAAGTTATTGGCACTGCGACCAGTGAAAATGGCGCTCAGGCGATCAGTGATTATTTA  225
 L  A  A  R  G  G  K  V  I  G  T  A  T  S  E  N  G  A  Q  A  I  S  D  Y  L   49

GGTGCCAAGGGCAAAGGTCTGATGTTGAATGTGACCGACCCGGCATCTATCGAATCTGTTCTGGAAAAAATTCGC  300
 G  A  N  G  K  G  L  M  L  N  V  T  D  P  A  S  I  E  S  V  L  E  K  I  R   74

GCAGAATTTGGTGAAGTGGATATCCTGGTCAATAATGCCGGTATCACTCGTGATAACCTGTTAATGCGCAATGAAA  375
 A  E  F  G  E  V  D  I  L  V  N  N  A  G  I  T  R  D  N  L  L  M  R  N  E   99

GATGAAGAGTGGAACGATATTATCGAAACCAACCTTTCATCTGTTTTCCGTCTGTCAAAAGCGGTAATGCCGCT  450
 D  E  E  W  N  D  I  I  E  T  N  L  S  S  V  F  R  L  S  K  A  V  M  R  A  124

ATGATGAAAAAAGCGTCATGGTCGTATTATCACTATCGGTTCTGTGGTTGGTACCATGGGAAATGGCGGTCAGGCC  525
 M  M  K  K  R  H  G  R  I  I  T  I  G  S  V  V  G  T  M  G  N  G  G  Q  A  149

AACTACGCTGCGGCCAAAGCGGGCTTGATCGGCTTCAGTAAATCACTGGCGCGCGAAGTTGCGTCACGCGGTATT  600
 N  Y  A  A  A  K  A  G  L  I  G  F  S  K  S  L  A  R  E  V  A  S  R  G  I  174

ACTGTAAACGTTGTTGCTCCGGGCTTTATTGAAACGGACATGACACGTGCGCTGAGCGATGACCAGCGTGCGGGT  675
 T  V  N  V  V  A  P  G  F  I  E  T  D  M  T  R  A  L  S  D  D  Q  R  A  G  198

ATCCTGCGCGCAGGTTCCTGCGGGTCGGCTCGGCGGCGCACAGGAAATCGCCAACGCGCGGTTGCATTCCTGGCATCC  750
 I  L  A  Q  V  P  A  G  R  L  G  G  A  Q  E  I  A  N  A  V  A  F  L  A  S  223

GACGAAGCAGCTTACATCACGGGTGAAACTTTGCATGTGAACGGCGGGGATGTACATGGTCTGACCGCGATTTGCA  825
 D  E  A  A  Y  I  T  G  E  T  L  H  V  N  G  G  M  Y  M  V  *  244

CAAAATGCTCATGTTGCGCGCAGTCTGCGTGGTTATGAGTAATAATTAGTGCAAAATGATTTGCGTTATTGGGGG  900
GTAAGGCCTCAAAATAACGTAAAATCGTGGTAAGACCTGCGGGGATTTAGTTGCAAATTTTTCAACATTTTATAC  975
ACTACGAAAACCATCGCGAAAGCGAGTTTTGATAGGAAATTTAAGAGTATGAGCACTATCGAGAACGCGTTAAG 1050
                                                M  S  T  I  E  R  V  K   9
                                                acpP
AAAATTATCGGCGAACAGCTGGGCGTTAAGCAGGAAGAAGTTACCAACAATGCTTCTTTCGTTGAAGACCTGGGC 1125
 K  I  I  G  E  Q  L  G  V  K  Q  E  E  V  T  N  N  A  S  F  V  E  D  L  G   34

GCGGATTCTCTTGACACCGTTGAGCTGGTAATGGCTCTGGAAGAAGAGTTTGATACTGAGATTCCGGACGAAGAA 1200
 A  D  S  L  D  T  V  E  L  V  M  A  L  E  E  E  F  D  T  E  I  P  D  E  E   59

GCTGAGAAAATCACCACCGTTCAGGCTGCCATTGATTACATCAACGGCCACCAGGCGTAAGTGAACATCTCCAGG 1275
 A  E  K  I  T  T  V  Q  A  A  I  D  Y  I  N  G  H  Q  A  *  ---  --  78

CGGTCGTTCGACCGCCTTGAGTTTTATCTTTTTGTCCCACTAGAATCATTTTTTCCCTCCCTGCAGGACAAACGT 1350
------>  <-------  ---
GTCTAAGCGGTCGTGTAGTTGTGACCGGA                                              1378
```

FIG. 1. **Physical map and sequence of the *fabG-acpP* region.** *Top panel*, a restriction map of the 2.6-kbp *SalI-BglII* genomic fragment is given on the *top line*. The outermost *PstI*, *KpnI*, and *EcoRI* sites are vector sites. The strategy used for DNA sequencing is shown *underneath* the *map*. *Arrows* indicate the extent and direction of sequencing. *bp*, base pairs. *Bottom panel*, nucleotide and deduced amino acid sequence of the *fabG* and *acpP* genes. A putative transcriptional terminator is indicated by *arrows*. The first 63 nucleotides encode the carboxyl terminus of malonyl transacylase (*fabD*).

The *acpP* gene was localized on the genetic map of *E. coli* by inserting a kanamycin resistance (Kan^R) determinant into pMR33 within a DNA sequence downstream of *acpP*. The Kan^R sequence was then transferred into the *E. coli* chromosome by homologous recombination (26) to give strain MR52. Conjugational mapping located the *acpP* gene between min 13 and 30, whereas P1-mediated transduction localized the gene to min 24 (98% linkage of the Kan^R insertion with the *zce-726*::Tn*10* insertion (27)). This location is very close to those we previously assigned to genes encoding two other fatty acid biosynthetic proteins, *fabD* and *fabF*, which encode malonyl-CoA-ACP transacylase and 3-ketoacyl-ACP synthase II, respectively (1, 19). It therefore, seemed probable that *acpP* was linked to these genes. Indeed, when phage P1 grown on the Kan^R insertion strain was used to transduce a *fabD* strain to Kan^R, 98% of the transductants were *fab*$^+$. The Kan^R insertion of strain MR52 could not be mapped in relation to *fabF* because the insertion of the Kan^R element resulted in a strain having a *fabF* phenotype.

fabF mutants have no growth phenotype unless the strain also has a lesion in the *fabB* gene that encodes 3-ketoacyl-ACP synthase I (1, 19). Mutants with a temperature-sensitive lesion in *fabB* (fabB^{ts}) fail to grow at 42 °C on the usual media but grow well if the medium is supplemented with oleate (or other appropriate unsaturated fatty acids). *fabF fabB*^{ts} double mutants fail to grow at 42 °C even when supplemented with oleate (due to defective synthesis of saturated fatty acids). We found that P1 cotransduction of a *fabB*^{ts} lesion into strain MR52 gave a *fabB*^{ts}*fabF* phenotype. Strain MR52 also showed other aspects of the *fabF* phenotype (19): (i) an increased level of palmitoleic acid and a decreased level of *cis*-vaccenic acid compared with the parental strain lacking the insertion; (ii) defective thermal regulation of fatty acid composition; (iii) lack of the 3-ketoacyl-ACP synthase II-ACP mixed disulfide in cell extracts. Thus, the Kan^R insertion of strain MR52 is either in *fabF* or is polar on *fabF* expression. We favor the former explanation since strain MR52 was unable to donate a functional *fabF* gene to a *fabB*^{ts}*fabF* strain via P1 transduction. The segment of DNA containing *acpP* was also located on the physical map of *E. coli*. The *acpP* DNA segment hybridized to phages 235 and 236 of the ordered miniset bank of Kohara and co-workers (28). Comparison of our restriction map to the physical map (28) placed the *acpP* gene at 1170 kbp, a site fully consistent with the genetic map location.

Given the close genetic linkage of *acpP*, *fabD*, and *fabF*, we examined the proteins encoded by the *acpP* plasmid to see if proteins the size of the *fabD* and *fabF* gene products (35 and 43 kDa, respectively) were encoded by the chromosomal insert of the plasmid. Analysis of the products of a maxicell (29) labeling procedure (which gives specific labeling of plasmid-encoded proteins) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the chromosomal insert encoded proteins of 20, 26.5, and 43 kDa in addition to the proteins encoded by the vector sequences (Fig. 2). The 20-kDa protein is ACP which migrates aberrantly (as though a

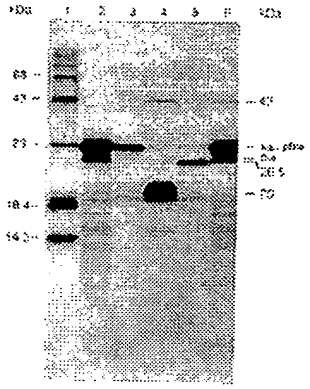

FIG. 2. **Maxicell analysis of plasmid-encoded proteins.** *Lane 1*, ^{14}C-labeled protein standards; *lane 2*, vector pTZ18R (21); *lane 3*, pMR62, a derivative of vector pACYC177 (20); *lane 4*, pMR24, which carries the entire 2.6-kbp chromosomal insert in pMR62 (Fig. 1); *lane 5*, pMR48, which carries the left-hand 1.1-kbp *Pst*I-*Pvu*II fragment (Fig. 1); and *lane 6*, pMR33 which contains the right-hand *Eco*RI-*Pvu*II fragment (Fig. 1). pMR48 and pMR33 are derived from vector pTZ19R (20). The 20- and 26.5-kDa proteins were the products of the *acpP* and *fabG* genes, respectively. *kan*, aminoglycoside phosphotransferase; *pBla*, the precursor of β-lactamase; *Bla*, β-lactamase.

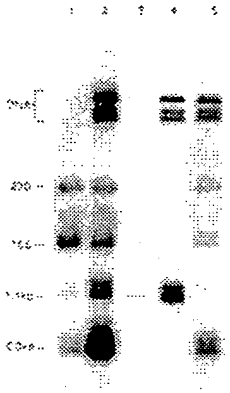

FIG. 4. **Northern blot analysis of *acpP* and *fabG* transcripts.** Whole cell lysates were electrophoresed on a 1% agarose/formaldyde gel, blotted, and probed (38). Lysates of strain F' M15 (*lane 1*) or F' M15A carrying either pMR24 (*lane 2*) or pMR36 (*lane 6*) were probed with the synthetic ACP gene. Plasmid pMR24 contains the intact *acpP-fabG* region whereas in pMR36 the *acpP* gene is inverted. *Lanes 3* and *4* are the same RNA samples of *lanes 1* and *2*, respectively, but the probe was the 0.4-kbp *Kpn*I-*Stu*I fragment (Fig. 1) specific for the *fabG* gene. Plasmid DNA (which is isolated with the RNA in the procedure used (38)), the cross-hybridizing rRNAs, and the size (in kb) of the two major transcripts are indicated at the *left margin*.

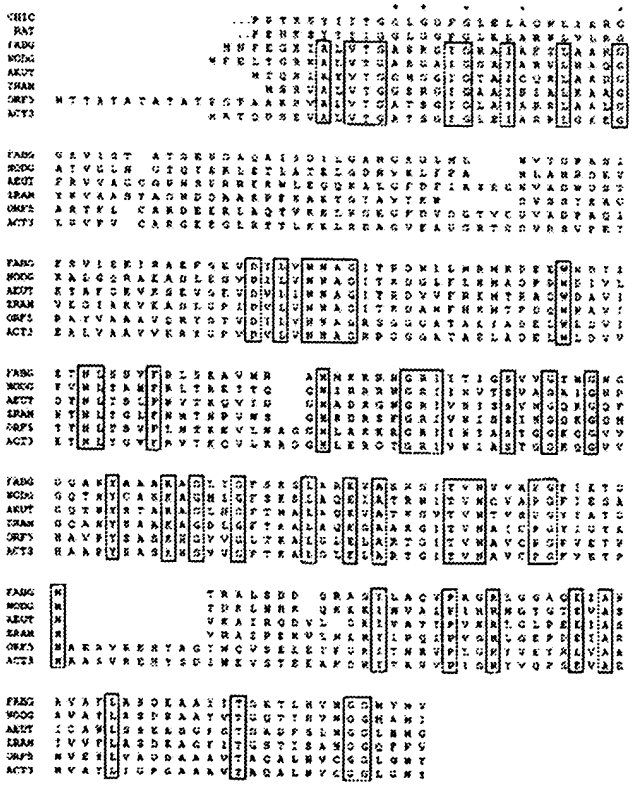

FIG. 3. **Comparison of the amino acid sequence of the *fabG* gene product with similar sequences.** Shown are the complete sequences for *E. coli fabG*, *R. meliloti nodG* (36), acetoacetyl-CoA reductases from *A. eutrophus* (*AEUT* (34)) and *Z. ramigera* (*ZRAM* (33)), *S. violaceoruber* ORF5 (7) and *S. coelicolor* actIII (7). Residues identical in all six sequences are *boxed*. Also shown is a portion of the ketoreductase domains from the rat (*RAT* (5)) and chicken (*CHIC* (35)) multifunctional fatty acid synthases. Amino acids which comprise a putative NADPH-dinucleotide fold are indicated by *asterisks*.

much larger protein) due to lower sodium dodecyl sulfate binding than the marker proteins (30). Deletion of the ACP sequence resulted in loss of the 20-kDa protein. Maxicell analysis of various subclones (Fig. 2) showed that the 43-kDa protein was encoded by a DNA segment located downstream of *acpP*, and hence, this protein may well represent a slightly truncated *fabF* gene product, 3-ketoacyl-ACP synthase II, a protein of 44 kDa (31). (The Kan^R insertion of strain MR52 would interrupt the synthesis of this protein.) The 26.5-kDa protein is encoded upstream of *acpP* but is not the *fabD* gene product since malonyl CoA-ACP transacylase has a molecular mass of 36.5 kDa (32). We have sequenced the DNA segment upstream of *acpP* and find an ORF that encodes a protein of 244 residues having a calculated molecular weight of 25,549 in good agreement with the maxicell results (Fig. 2). Comparison of the derived amino acid sequence of this ORF with those of GenBank showed a number of proteins with strong similarity to the ORF. The most definitive of these similarities was with two enzymes involved in poly-3-hydroxybutyrate synthesis in bacteria (33, 34). These are acetoacetyl-CoA reductases which reduce acetoacetyl-CoA (formed by condensation of two acetyl-CoA units) to the 3-hydroxybutryl-CoA used in polymer synthesis (33, 34). The ORF upstream of *acpP* showed 43 and 41% amino acid identity with the NADPH-specific acetoacetyl-CoA reductases of *Zoogloea ramigeria* (33) and *Alcaligenes eutrophus* (34), respectively (Fig. 3). We also found significant similarities to a segment of the large polyfunctional fatty acid synthase proteins of rat (5) and chicken (35). Strong similarities (40–53% amino acid identities) were also found to genes involved in polyketide synthesis in various *Streptomyces* (7) and to the *nodG* protein of *Rhizobium meliloti* (36) which may be involved in synthesis of acylated polysaccharides (10, 37). These relationships (Fig. 3) together with the presence of a plausible NADPH binding site in the upstream ORF and cotranscription of the ORF with *acpP* (see below) lead us to believe this ORF encodes a 3-ketoacyl-ACP reductase of fatty acid biosynthesis (13), a gene we term *fabG*.

The close juxtaposition of these coding sequences suggested

possible cotranscription of these genes. The maxicell results suggested promoters were present just upstream of both the *acpP* and *fabG* coding sequences, and this was confirmed by Northern blot analyses. Two chromosomal transcripts of about 0.3 and 1.1 kb were detected using the synthetic ACP gene as a probe (Fig. 4). A strain carrying pMR24 showed increased levels of both mRNA species. The 0.3-kb transcript has the capacity to encode ACP whereas the 1.1-kb transcript could encode both *acpP* and *fabG*. Only the 1.1-kb transcript was observed when a probe containing *fabG* sequences alone was used (Fig. 4). The relationship between the 0.3- and 1.1-kb transcripts was examined by inverting the *acpP* coding sequence within pMR24. When probed with the synthetic ACP gene, the 0.3-kb mRNA was the dominant transcript (Fig. 4). Therefore, the 0.3-kb mRNA seems a primary transcript and not a degradation product of the 1.1-kb mRNA. Thus, *acpP* is transcribed from two promoters. A strong promoter is located just upstream of the coding sequence, and a second is located upstream of the *fabG* sequence.

CONCLUSIONS

ACP is encoded by the *acpP* gene. Genes encoding other fatty acid biosynthetic genes lie both upstream and downstream of *acpP*. One downstream gene is *fabF*, encoding 3-ketoacyl-ACP synthase II, and several genes are located upstream. We have shown that *fabG* (which almost certainly encodes a 3-ketoacyl-ACP reductase) lies just upstream of *acpP* and is cotranscribed with *acpP*. In other work (41) we find that *fabD* encoding malonyl CoA-ACP transacylase is located just upstream of *fabG*. Indeed the first 63 bp of Fig. 1 encode the last 21 amino acids of *fabD* (41). Upstream of *fabD* lies another ORF (called *fabH*) that encodes 3-ketoacyl-ACP synthase III (40). Thus, the genes encoding several enzymes of fatty acid synthesis are clustered around the *acpP* gene and the toxicity of increased expression of *acpP* probably explains prior difficulties in cloning these genes. Our current map of this region (clockwise on the physical/genetic map) is *fabH-fabD-fabG-acpP-fabF*. The gene order has no obvious relationship to the order of protein domains in the polyfunctional fatty acid synthases of mammals or fungi.

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β-Ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths

Tung T. Hoang,† Sarah A. Sullivan,‡ John K. Cusick§ and Herbert P. Schweizer

Author for correspondence: Herbert P. Schweizer. Tel: +1 970 491 3536. Fax: +1 970 491 1815. e-mail: herbert.schweizer@colostate.edu

Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682A, USA

The two acyl-homoserine lactones (AHLs) *N*-(butyryl)-L-homoserine lactone and *N*-[3-oxododecanoyl]-L-homoserine lactone (3-oxo-C_{12}-HSL) are required for quorum sensing in *Pseudomonas aeruginosa*. These AHLs derive their invariant lactone rings from *S*-adenosylmethionine and their variable acyl chains from the cellular acyl-acyl carrier protein (ACP) pool. This reaction is catalysed by specific AHL synthases, which exhibit acyl chain specificity. Culture supernatants of *P. aeruginosa* contain multiple 3-oxo-AHLs that differ in their acyl chain lengths but their physiological role, if any, remains unknown. An *in vitro* fatty acid-3-oxo-AHL synthesis system was established utilizing purified *P. aeruginosa* Fab proteins, ACP and the LasI 3-oxo-AHL synthase. In the presence of excess protein, substrates and cofactors, this system produced almost exclusively 3-oxo-C_{12}-HSL. When the β-ketoacyl-ACP reductase (FabG) catalysed step was made rate-limiting by switching from the preferred NADPH cofactor to NADH, increased levels of short chain 3-oxo-AHLs were produced, presumably because shorter-chain ketoacyl-ACPs accumulated and thus became LasI substrates. Consistent with these *in vitro* observations, a *fabG*(Ts) mutant produced increased amounts of 3-oxo-AHLs *in vivo*. Thus, *in vitro* and *in vivo* evidence indicated that modulation of FabG activity of the fatty acid biosynthetic pathway may determine the acyl chain lengths of these 3-oxo-AHLs and that the LasI 3-oxo-AHL synthase is sufficient for their synthesis.

Keywords: *Pseudomonas*, homoserine lactone, fatty acid synthesis, synthase

INTRODUCTION

The expression of many extracellular *Pseudomonas aeruginosa* virulence factors (Passador *et al.*, 1993; Van Delden & Iglewski, 1998) and other cellular processes, such as biofilm maturation *in vitro* (Davies *et al.*, 1998) and biofilm formation in the lungs of cystic fibrosis patients (Singh *et al.*, 2000) are regulated in a cell-density-dependent manner by a process called cell-to-cell communication or quorum sensing. Cell-to-cell communication in *P. aeruginosa* involves the two acyl homoserine lactones (AHLs) *N*-(butyryl)-L-homoserine lactone (C_4-HSL) and *N*-[3-oxododecanoyl]-L-HSL (3-oxo-C_{12}-HSL). Although these two AHLs seem to be the main players involved in quorum sensing, *P. aeruginosa* produces other AHLs which differ by their acyl chain lengths but their physiological roles, if any, remain unclear. A quinolone signal (Pesci *et al.*, 1999) and perhaps cyclic peptides (Holden *et al.*, 1999) also seem to participate in some of these regulatory networks.

Several previous studies revealed that bacterial AHLs derive their invariant homoserine lactone rings from *S*-adenosyl methionine (SAM) and their variable acyl

†**Present address:** Department of Microbiology, University of Hawaii at Manoa, Honolulu, HI 96822, USA.

‡**Present address:** VWR International, 106 Gray Road, Suite D, Indianapolis, IN 46237, USA.

§**Present address:** National Jewish Hospital, 1400 Jackson Street, Denver, CO 80206, USA.

Abbreviations: ACP, acyl carrier protein; AHL, acyl homoserine lactone; C_4-HSL, *N*-(butyryl)-L-homoserine lactone; Fab, fatty acid biosynthesis; HSL, homoserine lactone; SAM, *S*-adenosyl methionine.

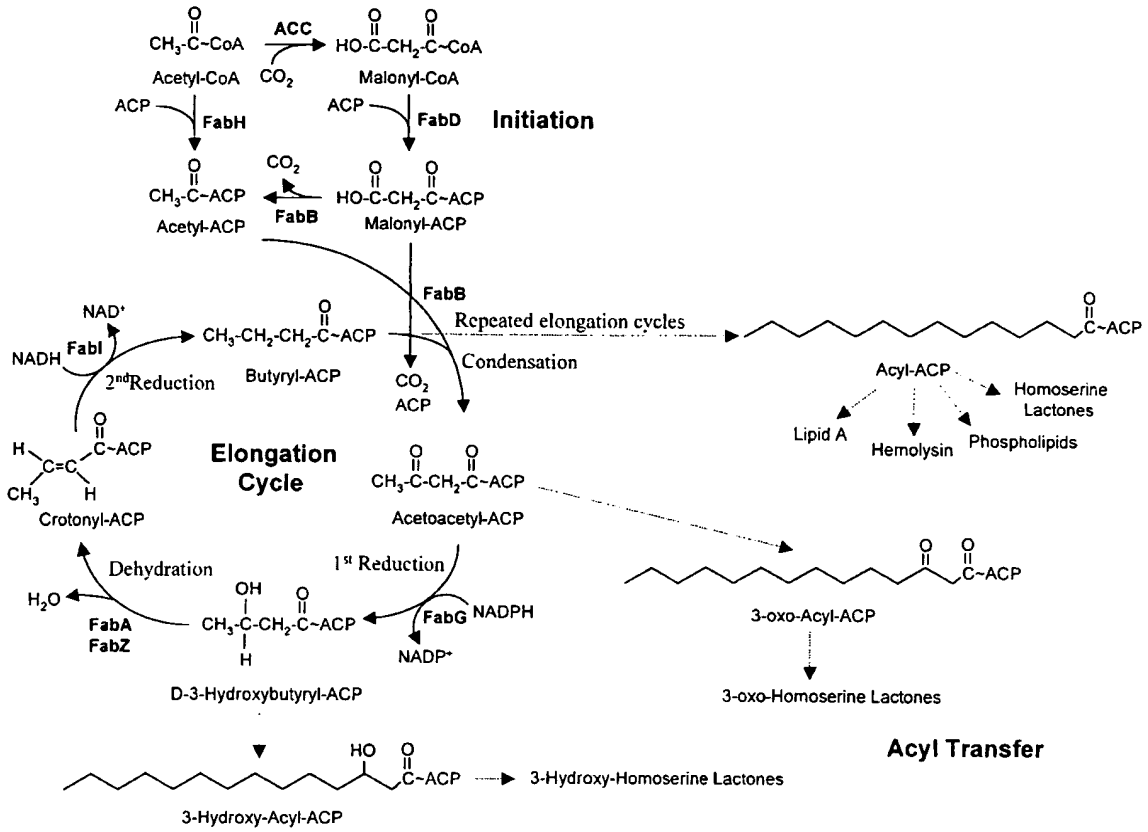

Fig. 1. Fatty acid biosynthesis in *P. aeruginosa*, and acyl-ACPs as acyl donors in cellular metabolism and AHL synthesis. There are several potential pathways to generate acetoacetyl-ACP and initiate fatty acid synthesis (Cronan & Rock, 1996) but not all are shown for the sake of clarity. In the reaction shown, which explains the *in vitro* system established in this study, malonyl-ACP is decarboxylated to acetyl-ACP by FabB, which then condenses these two molecules to acetoacetyl-ACP to initiate the cycle (Cronan & Rock, 1996). The malonyl-ACP is derived from malonyl-CoA by malonyl-CoA:ACP acyltransferase (FabD). Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalysed by FabB (β-ketoacyl ACP synthase I). The β-ketoacyl-ACP from the FabB reaction is reduced to a β-hydroxyacyl-ACP by FabG, a NADPH-dependent β-ketoacyl-ACP reductase. The subsequent dehydration step is catalysed by either FabA or FabZ, depending on the lengths of the acyl groups in the β-hydroxyacyl-ACP substrates. The final step involves reduction of the dehydratase product to an acyl-ACP via FabI, a NADH-dependent enoyl-ACP reductase. Subsequent cycles are initiated by a FabB-catalysed condensation of malonyl-ACP with acyl-ACP. For synthesis of 3-oxo-C_{12}-HSL, LasI utilizes the 3-oxo-dodecanoyl-ACP from the Fab pathway. Similarly, RhlI uses crotonyl-ACP for synthesis of C_4-HSL. Enzymes involved in 3-OH-AHL synthesis probably use D-3-hydroxy-ketoacyl-ACP substrates from the Fab cycle. Other biosynthetic pathways, including the phospholipid, lipopolysaccharide, haemolysin and other pathways, also use acyl-ACP intermediates. Other abbreviations: ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; FabH, β-ketoacyl ACP synthase III.

chains from the cellular acyl-ACP (acyl carrier protein) pool (Hoang & Schweizer, 1999; Moré *et al.*, 1996; Parsek *et al.*, 1999; Val & Cronan, 1998) (Fig. 1). Acyl chain specificity resides in critical amino acid residues within the AHL synthase sequences (Watson *et al.*, 2002). The AHL synthases (LasI for 3-oxo-C_{12}-HSL and RhlI for C_4-HSL) are sufficient for catalysis of the acyl transfer and lactonization reactions (Moré *et al.*, 1996; Parsek *et al.*, 1999; Hoang & Schweizer, 1999; Hoang *et al.*, 1999). *P. aeruginosa* culture supernatants contain 3-oxo-AHLs with various acyl chain lengths but their metabolic origins have not been elucidated. In this study, we attempted to elucidate the molecular basis for the synthesis of these 3-oxo-AHLs. Since LasI competes with NADPH-dependent β-ketoacyl-ACP reductase, FabG, for the 3-oxo-acyl-ACP precursors for synthesis of these 3-oxo-AHLs (Fig. 1), we reasoned that FabG activity may be a modulating factor determining acyl chain lengths in 3-oxo-AHLs. Because most Fab (fatty acid biosynthesis) enzymes, including FabG, are essential, conventional mutant analysis cannot be used to address their roles in cellular metabolism. To circumvent these problems, a complete *in vitro* Fab system using purified *Escherichia coli* Fab proteins and ACP was previously described and was shown to produce the types and distribution of acyl-ACP species found *in vivo* (Heath & Rock, 1996a, b). Since the *E. coli* and *P. aeruginosa* Fab systems are quite similar, we reasoned

that an *in vitro* Fab-3-oxo-AHL synthesis system could be used to explore FabG activity as a factor determining acyl chain lengths of 3-oxo-AHLs. To this end, we purified the *P. aeruginosa* Fab proteins as hexahistidine (H_6) fusion proteins and developed an *in vitro* Fab-AHL synthesis system by coupling them to purified LasI. Some of the observations made with the *in vitro* system were supported by preliminary *in vivo* data obtained with a conditional, temperature-sensitive *fabG*(Ts) mutant.

METHODS

Strains and growth media. *Escherichia coli* strains used in this study were DH5α (Liss, 1987), BL21(DE3) (Novagen), SA1503(DE3) (Hoang *et al.*, 1999) and the 3-oxo-C_{12}-HSL reporter strain MG4/pKDT17 (*lasR*$^+$ *lasB–lacZ*) (Schaefer *et al.*, 2000). The wild-type *P. aeruginosa* strain PAO1 was previously described (Watson & Holloway, 1978). *P. aeruginosa* strain 4 is a clinical wound isolate from the Glaxo SmithKline collection and is similar to PAO1 in terms of extracellular protein profiles, exoenzyme S production and nucleotide sequences. The *fabG*(Ts) mutant ts-67 was derived from strain 4 by Dr J. Huang (Collegeville, PA, USA) at Glaxo SmithKline and strain ts-67R1 is a revertant of strain ts-67. The *Agrobacterium tumefaciens* strains NTL4/pZLR4 (containing *traR* and *traG*::*lacZ*) and NT1/pTiC58Δ*accR* were from S. Farrand (University of Illinois, Urbana, USA). The *Erwinia carotovora* strain EC14 was previously described (Schweizer, 1994). Unless otherwise indicated, bacterial strains were grown in LB medium (Difco), which for plasmid maintenance in *E. coli* was supplemented with 100 μg ampicillin ml^{-1} and/or 25 μg chloramphenicol ml^{-1}.

Construction of expression vectors and affinity purification of proteins. The coding sequences for the individual enzymes were PCR amplified from PAO1 genomic DNA utilizing *Taq* polymerase and previously described conditions (Hoang & Schweizer, 1999; Hoang *et al.*, 1998). The general strategy involved the use of a forward primer that incorporated an *Nde*I restriction site at the start codon of the respective gene and a reverse primer that incorporated a *Bam*HI restriction site after the stop codon of the same gene (Table 1). The gel-purified (QIAquick gel extraction kit; Qiagen) PCR fragments were digested with *Nde*I/*Bam*HI and then ligated between the same sites of pET-15b (Novagen). Since *fabG* contained a *Bam*HI site, the reverse primer incorporated a *Bgl*II site, which allowed subcloning into the *Bam*HI site of pET-15b. Standard molecular biological techniques were used (Sambrook & Russell, 2001). Subcloning into pET-15b yielded the expression vectors pPS837 (FabB), pPS980 (FabG), pPS998 (FabH) and pPS937 (FabZ). For FabA, the PCR fragment was first cloned into the TA cloning vector pGEM-T (Promega) to yield pPS847. An *Nde*I–*Bam*HI fragment derived from this plasmid was then subcloned between the same sites of pET-15b (Novagen) to yield the FabA expression vector pPS848. For expression of the resulting proteins with NH_2-terminal hexahistidine (H_6) tags, the plasmids were transformed into BL21(DE3) (Novagen). Screening of H_6-Fab protein-expressing transformants, cell lysis and purification of the soluble fusion proteins on Ni^{2+} agarose affinity columns (Qiagen) was performed as previously described (Hoang *et al.*, 1999), except for FabD. Since FabD eluted from the columns with 40 mM imidazole, washing of the column was done with 30 bed vols buffer with 20 mM imidazole.

ACP was purified via an intein chitin-binding domain fusion protein as previously described (Kutchma *et al.*, 1999), except that it was coexpressed with acyl-ACP synthase (AcpS) to maximize holo-ACP formation. To this end, the AcpS expressing pPS1118 was constructed by subcloning the *acpS* gene from *E. coli* on a 470 bp *Ase*I–*Hin*dIII fragment from pDPJ (Lambalot & Walsh, 1995) between the same sites of pACYC184 (Chang & Cohen, 1978). For coexpression of ACP and AcpS, the expression strain was grown in LB + ampicillin + chloramphenicol medium to maintain the *acpP*- and *acpS*-containing plasmids. H_6-LasI was purified using a published procedure (Hoang *et al.*, 1999).

Protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad) and BSA as the standard. Proteins were analysed by electrophoresis on 0·1% SDS-10% polyacrylamide gels (SDS-PAGE) (Makowski & Ramsby, 1993) and visualized by staining with Coomassie Brilliant Blue R-250 (Chen *et al.*, 1993).

Complementation assays. The coding sequences for the H_6-tagged FabA, FabB and FabD proteins were subcloned into the

Table 1. List of PCR primers

The forward primers incorporated an *Nde*I restriction site (underlined) at the start codon of the respective genes. In most cases, the reverse primers incorporated a *Bam*HI restriction site (underlined) after the stop codon with the exception of FabG (reverse), which incorporated a *Bgl*II site after the *fabG* stop codon.

Name	Sequence (5′–3′)
FabA (forward)	TCATATGACCAAACAACACGCCTTCAC
FabA (reverse)	GGATCCCCCTTAGAAGCTGTCAGTGGAG
FabB (forward)	TCCATATGCGTCGCGTCGTTATCACCGGTC
FabB (reverse)	ATGGATCCAATCAACCCTGCCAGCGCTTGAGGA
FabG (forward)	TGACATATGCCGCGCGCCGCCGTGGTCT
FabG (reverse)	GACAGATCTTATGACAGACCCGAGAAAGGTAAC
FabH (forward)	TGACATATGCCGCGCGCCGCCGTGGTCT
FabH (reverse)	GTGGATCCCTCTTCAGTCCATTGTCGG
FabZ (forward)	CCTCATATGATGGACATCAACGAGATTCG
FabZ (reverse)	GAGGATCCATCAAACTCATAGTTTGCGT

broad-host-range vector pUCP21T (Schweizer *et al.*, 1996) on *Bam*HI–*Xba*I fragments. Subcloning between the *Bam*HI and *Xba*I sites of pUCP21T placed the H_6-Fab coding sequences in the correct transcriptional orientation with respect to the *lac* promoter contained on this cloning vector and yielded pPS1013 (H_6-FabA), pPS1025 (H_6-FabB) and pPS1019 (H_6-FabD). To test for expression of functional H_6-FabA and H_6-FabB proteins, pPS1013 and pPS1025 were transformed (Hoang *et al.*, 1998) into strain PAO191 (*fabA*) and PAO192 (*fabB*) (Hoang & Schweizer, 1997), respectively. Since FabA and FabB are required for unsaturated fatty acid synthesis, PAO191 and PAO192 will not grow at 42 °C unless supplemented with oleic acid or complemented with either a FabA- or FabB-expressing plasmid. Complementation was therefore scored as the ability to grow at 42 °C on RB medium without oleate supplementation (Hoang & Schweizer, 1997). To test for expression of a functional H_6-FabD protein, pPS1019 was transformed into the *fabD*(Ts) mutant PAO204 (Kutchma *et al.*, 1999). Successful complementation was scored as the ability of the transformants to grow on LB plates at 42 °C. In all instances, strains were transformed with pUCP21T as a negative control.

Reconstitution of the Fab-AHL pathway and extraction of 3-oxo-acyl-HSLs. Complete reactions (total volume 500 µl) contained buffer [10 mM Tris/HCl (pH 7·4), 330 mM NaCl, 15 %, w/v, glycerol, 0·7 mM DTT, 2 mM EDTA, 25 mM $MgSO_4$, 0·1 mM $FeSO_4$] (Moré *et al.*, 1996), 2 µg ACP, 1 µg each FabA, FabB, FabD, FabH, FabI and FabZ, 0·5 µg FabG, 5 µg LasI, 0·25 mM SAM, 0·08 mM acetylCoA, 0·8 mM malonyl-CoA and 0·6 mM each NADH and NADPH (substrates and cofactors were obtained from Sigma). Reactions were incubated at 37 °C for 1 h and extracted three times with 250 µl ethyl acetate. Extracted AHLs were dried by rotary vacuum evaporation and resuspended in 20 µl acetonitrile. For detection of fractions containing AHLs, 5–10 µl each fraction was spotted on a C_{18} reverse-phase TLC plate (Whatman) and the plates were dried at 37 °C for 15 min before being overlaid with the detection strain. For TLC analysis of AHL fractions, the plates were developed in 60 % methanol in water (v/v) and then dried for 20 min at 37 °C prior to being overlaid with the detection strain.

Detection, identification and quantification of AHLs. *A. tumefaciens* reporter strain NTL4/pZLR4 was grown at 30 °C for 48 h in M9 medium (Miller, 1992) with 1 mM $MgSO_4$, 0·1 mM $CaCl_2$, 0·6 % glucose and 30 µg gentamicin ml^{-1} (Shaw *et al.*, 1997). Cells were harvested and resuspended in warm (~45 °C) fresh M9 medium with 0·4 % agar, 1 mM $MgSO_4$, 0·1 mM $CaCl_2$, 0·6 % glucose and 40 µg X-Gal ml^{-1}. This suspension was used immediately to overlay the TLC plates. The presence of AHLs was usually evident by the appearance of blue spots after incubation at room temperature for 36–48 h. Synthetic 3-oxo-C_{12}-HSL, and bacterial-derived 3-oxo-C_8-HSL and 3-oxo-C_6-HSL were included as standards. The latter two were extracted from 10 ml stationary-phase clarified culture supernatants of *A. tumefaciens* strain NT1/pTiC58Δ*accR* or *Erw. carotovora* strain EC14, respectively, using a previously described method (Shaw *et al.*, 1997). The concentrations of 3-oxo-C_{12}-HSL were estimated utilizing the *Esc. coli* reporter strain MG4/pKDT17 (*lasR*$^+$ *lasB–lacZ*) as previously described (Schaefer *et al.*, 2000) and by using a dilution series of synthetic 3-oxo-C_{12}-HSL to establish a standard curve. For determination of HSL levels in the supernatants of the *fabG*(Ts) mutant ts-67, its parental strain 4 and the ts-67R1 revertant of strain ts-67, the strains were grown in LB medium until the cultures reached an optical density of ~1·6 (600 nm). The pH in the cultures was monitored to avoid excess alkalinization of the medium since AHLs are very unstable at alkaline pH values (Schaefer *et al.*, 2000). Aliquots (1 ml) were harvested by centrifugation. The supernatants were extracted three times with 1 ml acidified ethyl acetate (ethyl acetate containing 0·1 ml glacial acetic acid per litre), dried and suspended in 200 µl acidified ethyl acetate. For detection of fractions containing 3-oxo-HSLs, 10 µl each fraction was spotted on a C_{18} reverse-phase TLC plate. The plates were processed as described above and then overlaid with the *A. tumefaciens* detection strain.

RESULTS AND DISCUSSION

Purification and *in vivo* activity of Fab proteins

Our initial goal was to set up a complete *in vitro* Fab-AHL synthesis system using only *P. aeruginosa* proteins by coupling purified Fab enzymes to LasI 3-oxo-AHL synthase. Since we previously described the purification and activity of ACP (Kutchma *et al.*, 1999), FabD (Kutchma *et al.*, 1999), FabI (Hoang & Schweizer, 1999) and LasI (Hoang *et al.*, 1999), we still needed to purify FabA, FabB, FabG, FabH and FabZ, assuming that all of these proteins are needed to synthesize acyl-ACPs from acetyl-CoA and malonyl-CoA (Fig. 1). Using non-denaturing conditions and metal chelation affinity chromatography, all Fab proteins were purified to near homogeneity after overexpression in *E. coli* (Fig. 2). When expressed *in vivo* from the *lac* promoter, the genes encoding the H_6-tagged FabA, FabB and FabD proteins complemented the corresponding *P. aeruginosa* mutations, indicating that the constructs expressed enzymically active H_6-Fab proteins. We previously showed that

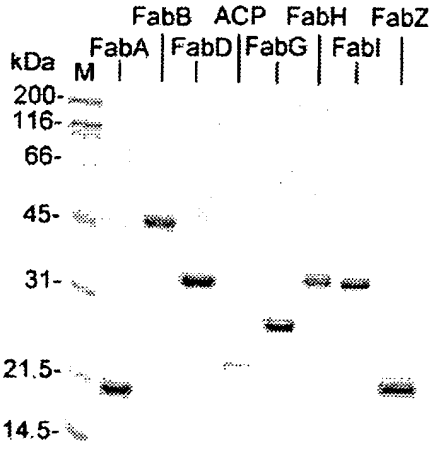

Fig. 2. Gel electrophoretic analysis of purified proteins. Samples of purified ACP and the various Fab proteins were analysed by electrophoresis on a 0·1 % SDS-13 % PAGE. The gel was stained with Coomassie blue. All proteins, except ACP, were purified with NH_2-terminal H_6-tag containing extensions. ACP was purified in its native form via an ACP-intein chitin-binding domain fusion protein. The sizes of protein markers (M) from Bio-Rad are indicated in kDa and were (top to bottom): myosin, β-galactosidase, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme.

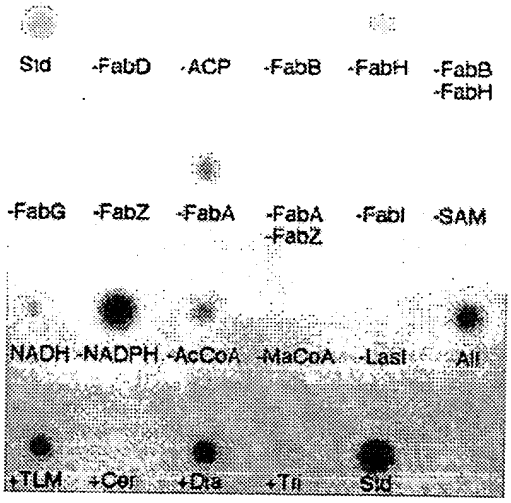

Fig. 3. Enzymic synthesis of 3-oxo-AHLs in a reconstituted enzyme system. Ethyl acetate extracts of reactions were analysed for the presence of 3-oxo-AHLs by spotting samples on a C_{18}-reverse-phase TLC plate and overlaying it with the *A. tumefaciens* NTL4(pZLR4) detection strain in the presence of X-Gal. Spots indicate the presence of 3-oxo-acyl-HSLs and a 3-oxo-C_{12}-HSL standard (Std). The complete reaction (All) contained ACP, FabA, FabB, FabD, FabG, FabH, FabI, FabZ, LasI, SAM, acetyl-CoA, malonyl-CoA, and NADH and NADPH. The other reactions lacked the indicated enzymes, substrates or co-factors. Some reactions contained 50 μM of the Fab inhibitors thiolactomycin (TLM), cerulenin (Cer), diazaborine (Dia) or triclosan (Tri). Enzyme abbreviations are explained in Fig. 1. Other abbreviations: AcCoA, acetyl-CoA; MaCoA, malonyl-CoA.

expressed H_6-FabI complemented an *E. coli fabI*(Ts) mutant and was enzymically active (Hoang & Schweizer, 1999). Complementation experiments were not possible for FabH and FabZ since no mutants were available.

Establishment of an *in vitro* Fab-3-oxo-AHL synthesis system

The Fab-3-oxo-AHL pathway was reconstituted *in vitro* and biologically active 3-oxo-AHLs were detected using an *A. tumefaciens* indicator strain (Fig. 3). The results showed that the minimal Fab-3-oxo-AHL biosynthetic pathway consists of ACP, FabB, FabD, FabG, FabI, FabZ and LasI. Essential metabolites included malonyl-CoA and SAM. Lesser amounts of 3-oxo-AHLs were produced when acetyl-CoA and NADH were omitted. While our experiments confirmed the previously established importance of some components of the Fab system in AHL synthesis, i.e. the dependency on ACP, metabolites and cofactors (Moré *et al.*, 1996; Parsek *et al.*, 1999; Val & Cronan, 1998), the minimal pathway was to date unknown and could not have been determined without establishing the experimental system described in this study. The *in vitro* system also allowed an assessment of the relative contribution of the seemingly redundant components of the Fab system.

Synthases. Since FabB is the major condensing enzyme, it was essential for AHL formation from malonyl-CoA. In contrast, FabH was not required presumably since FabB can decarboxylate malonyl-ACP to acetyl-ACP and then condenses these two molecules to initiate the cycle without FabH (Fig. 1), as has been suggested for *E. coli* FabB (Cronan & Rock, 1996). This would also explain the formation of 3-oxo-AHLs in the reactions containing no acetyl-CoA.

Dehydratases. Of the two dehydratases, only FabZ was essential for AHL formation but not FabA. This is probably due to the fact that FabZ is mostly required in the initial cycles since its *E. coli* counterpart has greatest affinity for C_4–C_8 β-hydroxyacyl-ACP intermediates, but can use substrates with longer acyl chains (Heath & Rock, 1996a). In contrast, *E. coli* FabA acts preferably on C_{10}–C_{14} β-hydroxyacyl-ACP intermediates.

Reductants. Exclusion of NADH led to detectable AHL production but at much reduced levels. Since NADH is the reductant preferred by FabI (Hoang & Schweizer, 1999), this result indicates that FabI can utilize NADPH but that this step becomes rate-limiting in the absence of NADH.

When the known Fab inhibitors cerulenin, triclosan, diazoborine and thiolactomycin were added to the reaction mixture, only cerulenin and triclosan efficiently inhibited 3-oxo-AHL formation at the concentration tested (50 μM). For unknown reasons, at the same concentrations, thiolactomycin and diazoborine had little effect but from other experiments we suspected that these two antimicrobials, which are not available commercially, had lost much of their activities during storage (data not shown).

Nature of AHL molecules synthesized *in vitro*

TLC analysis (Fig. 4) was used to identify AHL species contained in representative positive reactions shown in Fig. 3. The analysis showed that reactions containing all essential components of the Fab-3-oxo-AHL synthesis system almost exclusively yielded 3-oxo-C_{12}-HSL, and only minute amounts of shorter chain 3-oxo-AHLs were discernible. Conversely, in the absence of NADPH but presence of NADH, LasI synthesized hardly any 3-oxo-C_{12}-HSL but larger amounts of 3-oxo-C_{10}-HSL and 3-oxo-C_8-HSL, and lesser amounts of 3-oxo-C_6-HSL (lane labelled -NADPH). Since 3-oxo-C_8-HSL is the cognate *A. tumefaciens* AHL, its spot size is not indicative of a higher quantity of 3-oxo-C_8-HSL relative to the other 3-oxo-AHLs, but rather indicates a better response to its native AHL. According to the pathway model (Fig. 1), LasI and FabG compete for 3-oxo-acyl-ACP substrates from the Fab system. Although FabG can utilize NADH, NADPH is its preferred cofactor and in its absence the FabG-catalysed reduction step becomes rate limiting, leading to accumulation of shorter chain 3-oxo-acyl-

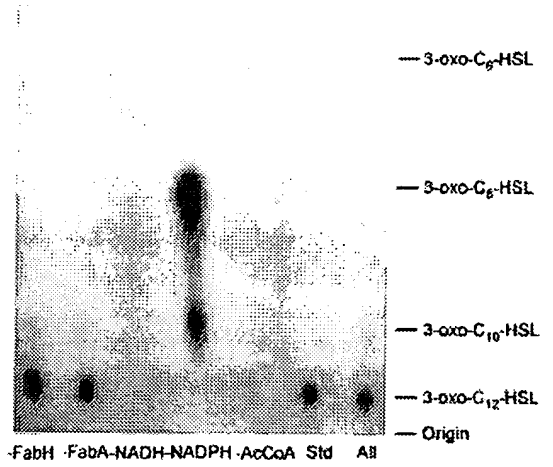

Fig. 4. Identification of AHLs produced in *in vitro* synthesis reactions. Extracted and concentrated products from selected reactions shown in Fig. 3 were spotted onto a C_{18}-reverse phase TLC plate. The plate was developed with 60% (v/v) methanol in water and overlaid with the *A. tumefaciens* NTL4(pZLR4) detection strain in the presence of X-Gal. All, complete reaction mixtures contained ACP, FabA, FabB, FabD, FabG, FabH, FabI, FabZ, LasI, SAM, acetyl-CoA, malonyl-CoA, and NADH and NADPH; other reactions lacked the indicated enzymes, substrates or co-factors. The relative mobility of known 3-oxo-acyl-HSLs, analysed on the same TLC plate but in a portion that is not shown, and the sample origin are marked on the right.

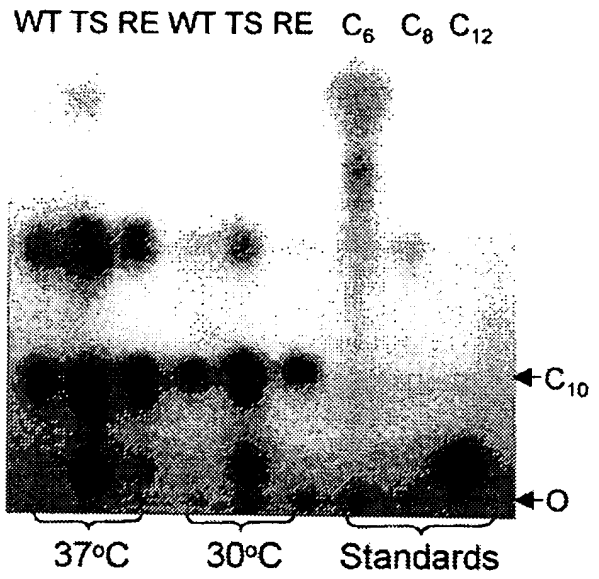

Fig. 5. Identification of 3-oxo-AHLs produced by a *fabG*(Ts) strain, its parent and a revertant. AHLs were extracted from cells grown at the indicated temperatures and samples of the concentrated reaction products were spotted onto a C_{18}-reverse phase TLC plate. The plate was developed with 60% (v/v) methanol in water and overlaid with the *A. tumefaciens* NTL4(pZLR4) detection strain in the presence of X-Gal. Samples analysed were from wild-type strain 4 (WT), its *fabG*(Ts) derivative (TS) and a revertant that contained a restored wild-type *fabG* sequence (RE). Standards included 3-oxo-C_6-HSL (C_6), 3-oxo-C_8-HSL (C_8) and 3-oxo-C_{12}-HSL (C_{12}). The relative mobility of 3-oxo-C_{10}-HSL (C_{10}), for which no standard was available, and the origin (O) are marked on the right.

ACPs. This now enables LasI to compete for the shorter-chain 3-oxo-acyl-ACP substrates and use them for synthesis of the corresponding shorter chain 3-oxo-AHLs. These results also proved that LasI alone is sufficient for synthesis of the shorter chain 3-oxo-AHLs found in *P. aeruginosa* culture supernatants.

A *fabG*(Ts) mutant is altered in 3-oxo-AHL production

A *fabG*(Ts) mutant was used to obtain preliminary *in vivo* experimental evidence for some of the *in vitro* observations. To examine whether altered FabG activity influenced 3-oxo-AHL production *in vivo*, AHL formation was analysed in a *fabG*(Ts) mutant grown in LB medium at permissive temperature (30 °C) and 37 °C, a temperature that is close to non-permissive (38 °C or higher). The *fabG*(Ts) mutant produced elevated levels of all 3-oxo-AHLs at both temperatures, most notably 3-oxo-C_6-HSL which under these experimental conditions was undetectable in supernatants obtained from wild-type and revertant strains, respectively (Fig. 5). Whereas the parental wild-type and the revertant strain produced levels of 3-oxo-C_{12}-HSL that remained nearly constant over the temperature range examined, the *fabG*(Ts) strain produced elevated levels of this 3-oxo-AHL, which increased with increasing temperatures (Table 2). These increasing 3-oxo-C_{12}-HSL levels were paralleled with a slight decrease in growth rates of the *fabG*(Ts) mutant as the temperature increased. The doubling times at 37 °C were 24 min for wild-type and revertant, and 36 min for the *fabG*(Ts) mutant. Similar observations to those presented in Fig. 5 and Table 2 were made when AHLs were extracted from cultures grown to lesser cell densities (data not shown). The most plausible explanation for these observations is that even at permissive temperatures the *fabG*(Ts) strain produces a FabG protein whose reductase activity is decreased when compared to wild-type or revertant FabG. Decreased FabG activity would lead to an increase in the intracellular 3-oxo-acyl-ACP pools, enabling LasI to compete for these substrates, ultimately resulting in increased 3-oxo-acyl-HSL levels.

Conclusions

The 3-oxo-AHLs normally found in *P. aeruginosa* culture supernatants contain acyl chains of 6–12 carbons (Shaw *et al.*, 1997) and the relative abundances of different 3-oxo-AHLs change during growth. The results obtained with our *in vitro* system gave the first clues that modulation of FabG activity by substrate and/or cofactor availability may at least partially explain these observations. In the presence of LasI, this AHL synthase

Table 2. Estimation of 3-oxo-C_{12}-HSL production by a *fabG*(Ts) mutant, its parent and a revertant

The strains were grown in LB medium to OD_{600} ~1·6 at the indicated temperatures. One millilitre aliquots were harvested by centrifugation. The supernatants were extracted three times with 1 ml acidified ethyl acetate, dried and suspended in 200 µl acidified ethyl acetate. The concentrations of 3-oxo-C_{12}-HSL were estimated using the *E. coli* reporter strain MG4/pKDT17 (*lasR*$^+$ *lasB–lacZ*) and a dilution series of synthetic 3-oxo-C_{12}-HSL to establish a standard curve. The values shown are the means ± standard deviations of triplicate measurements.

Strain	Extracellular 3-oxo-C_{12}-HSL (nM)		
	22 °C	30 °C	37 °C
4 (wild-type)	48·1 ± 8·8	39·3 ± 15·0	42·6 ± 15·4
ts-67 [*fabG*(Ts)]	252 ± 88·7	301 ± 79·8	487 ± 133
ts-67R1 (revertant to wild-type)	55·6 ± 10·7	47·1 ± 25·4	26·4 ± 7·39

and FabG compete for 3-oxo-acyl-ACP substrates from the fatty acid biosynthetic pathway. When FabG activity is high, turnover of the short chain 3-oxo-acyl-ACP substrates is rapid and LasI cannot compete for them, presumably because its affinity for them is lower than that of FabG. Once the acyl chain length reaches 12 carbons, LasI efficiently competes for the 3-oxo-C_{12}-ACP, resulting in synthesis of 3-oxo-C_{12}-HSL. When the FabG catalysed step becomes rate limiting, as mimicked in our experimental system by switching cofactors from the preferred NADPH to NADH, accumulation of shorter chain 3-oxo-acyl-ACPs results. This enables LasI to compete for these shorter-chain 3-oxo-acyl-ACP substrates and use them for synthesis of the corresponding shorter chain 3-oxo-AHLs. This explains why in the absence of NADPH only minute amounts of 3-oxo-C_{12}-HSL were synthesized in the *in vitro* reactions, while the levels of 3-oxo-C_8-HSL and 3-oxo-C_{10}-HSL were greatly elevated (Fig. 4). Consistent with these observations and conclusions, a *fabG*(Ts) mutant produced overall elevated levels of 3-oxo-AHLs, especially when it was grown at increasing temperatures (Fig. 5; Table 2), presumably since the respective 3-oxo-acyl-ACPs become available for LasI as the growth rate and therefore the demand for fatty acids for other biosynthetic processes decreases. The potential physiological relevance of 3-oxo-AHLs in *P. aeruginosa* other than 3-oxo-C_{12}-HSL, and the regulation of their relative abundances during cellular growth by modulation of FabG activity is currently unclear and awaits further investigation. FabG activity may be controlled at the genetic level (e.g. via transcriptional regulation of *fabG*) or at the protein level (via substrate allosteric effects).

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Transcriptional Analysis of Essential Genes of the *Escherichia coli* Fatty Acid Biosynthesis Gene Cluster by Functional Replacement with the Analogous *Salmonella typhimurium* Gene Cluster

YAN ZHANG[1] AND JOHN E. CRONAN, JR.[1,2]*

Departments of Microbiology[1] and Biochemistry,[2] University of Illinois, Urbana, Illinois 61801

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The genes encoding several key fatty acid biosynthetic enzymes (called the *fab* cluster) are clustered in the order *plsX-fabH-fabD-fabG-acpP-fabF* at min 24 of the *Escherichia coli* chromosome. A difficulty in analysis of the *fab* cluster by the polar allele duplication approach (Y. Zhang and J. E. Cronan, Jr., J. Bacteriol. 178:3614–3620, 1996) is that several of these genes are essential for the growth of *E. coli*. We overcame this complication by use of the *fab* gene cluster of *Salmonella typhimurium*, a close relative of *E. coli*, to provide functions necessary for growth. The *S. typhimurium fab* cluster was isolated by complementation of an *E. coli fabD* mutant and was found to encode proteins with >94% homology to those of *E. coli*. However, the *S. typhimurium* sequences cannot recombine with the *E. coli* sequences required to direct polar allele duplication via homologous recombination. Using this approach, we found that although approximately 60% of the *plsX* transcripts initiate at promoters located far upstream and include the upstream *rpmF* ribosomal protein gene, a promoter located upstream of the *plsX* coding sequence (probably within the upstream gene, *rpmF*) is sufficient for normal growth. We have also found that the *fabG* gene is obligatorily cotranscribed with upstream genes. Insertion of a transcription terminator cassette (Ω-Cm cassette) between the *fabD* and *fabG* genes of the *E. coli* chromosome abolished *fabG* transcription and blocked cell growth, thus providing the first indication that *fabG* is an essential gene. Insertion of the Ω-Cm cassette between *fabH* and *fabD* caused greatly decreased transcription of the *fabD* and *fabG* genes and slower cellular growth, indicating that *fabD* has only a weak promoter(s).

The bacterial fatty acid biosynthetic pathway is a type II, or disassociated-enzyme, system, where each of the reactions of the pathway is catalyzed by a discrete cytoplasmic enzyme. Fatty acid biosynthesis in *Escherichia coli* is the paradigm type II system, and much has been learned about the pathway in recent years (12, 25). Recent work has shown that about half of the fatty acid biosynthesis (*fab*) genes are clustered as a set of contiguous genes at min 24 of the *Escherichia coli* chromosome in the order *fabH-fabD-fabG-acpP-fabF* (4, 19, 22, 33), whereas the rest of the *fab* genes are scattered around the chromosome as separately transcribed genes (12). The proteins encoded by the genes of the cluster are PlsX, β-ketoacyl-acyl carrier protein (ACP) synthase III, malonyl-coenzyme A (CoA):ACP transacylase, β-ketoacyl-ACP reductase, ACP, and β-ketoacyl-ACP synthase II, respectively. We consider the *plsX* gene (located immediately upstream of *fabH*) to be part of the *E. coli* cluster due to its role (albeit poorly understood) in phospholipid biosynthesis (10). The *plsX* phenotype is defined by a single mutant allele, *plsX50*, which confers *sn*-glycerol 3-phosphate auxotrophy on strains carrying mutations in *plsB*, the gene that encodes *sn*-glycerol 3-phosphate acyltransferase, the first enzyme of phospholipid synthesis. The cluster is delimited upstream by the *rpmF* gene, encoding the L32 ribosomal protein (19), and downstream by a gene (*pabC*) involved in *p*-aminobenzoic acid synthesis (7).

Similar *fab* gene clusters have recently been reported in other bacteria: *Haemophilus influenzae* Rd (*fabH-fabD-fabG-acpP*) (5), *Vibrio harveyi* (*fabD-fabG-acpP-fabF*) (27), and *Rhodobacter capsulatus* (*plsX-fabH*) (3). The recently completed genomic sequence of *Helicobacter pylori* also contains *fab* cluster homologs (31). However, the *fab* cluster homologs of *H. pylori* are split relative to the *fab* cluster genes of *E. coli*. The *H. pylori* genome contains adjacent *plsX* and *fabH* genes, with a ribosomal protein gene (*rpmF*) located upstream of *plsX*, as seen in *R. capsulatus*, while the remainder of the genes found in the *E. coli fab* cluster, *fabD*, *fabG*, *acpP*, and *fabF*, are clustered with *accA* (which encodes an acetyl-CoA carboxylase subunit) at a location 200 kb removed from the first cluster, with another ribosomal protein gene (*rps21*) located upstream of *fabD*. Among gram-positive bacteria, similar *fab* gene clusters have been reported in *Bacillus subtilis* (*plsX-fabD-fabG-acpP*) (17) and *Streptomyces glaucescens* (*fabD-fabH-acpP-fabB*; note that *acpP* was called *fabC* in this organism and that the last gene is as closely homologous to *E. coli fabF* as to *E. coli fabB*) (28).

Although all the proteins (except PlsX) encoded by the genes of the *E. coli fab* gene cluster have been extensively studied, the transcription and regulation of these genes have only recently been investigated (20, 21, 36). Podkovyrov and Larson (20) reported promoter probe studies suggesting that the *rpmF-plsX* genes are cotranscribed, that several promoters are present, and that some of these transcripts may continue into the *fabHDG* genes (20). However, these results were obtained with transcriptional fusions carried on multicopy plasmids and have not been confirmed by direct mapping of chromosomal transcription, nor has the physiological relevance of the various promoters been determined. These workers have also reported the presence of a promoter located within the *plsX* coding sequence that reads through downstream *fab* genes (21).

* Corresponding author. Mailing address: B103 Chemical and Life Sciences Laboratory, 601 South Goodwin Ave., Urbana, IL 61801. Phone: (217) 333-0425. Fax: (217) 244-6697. E-mail: j-cronan@uiuc.edu.

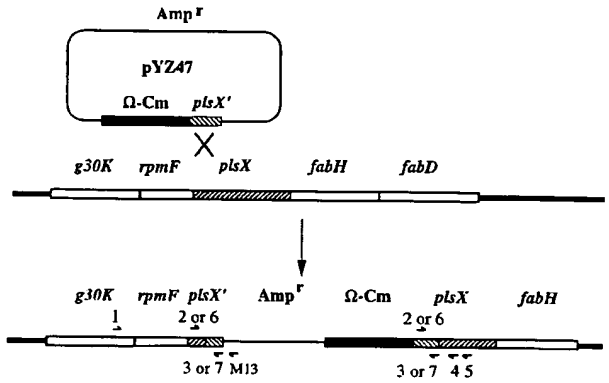

FIG. 1. Polar allele duplication of *plsX*. Plasmid pYZ47 was transformed into strain YZ133 (strain UB1005 harboring plasmid pYZ53), followed by selection for transformants resistant to ampicillin, chloramphenicol, and kanamycin. Plasmid pYZ47 (which is unable to replicate in this strain) integrated into the *E. coli* chromosome in a single-crossover event via homologous recombination between the truncated *plsX'* gene of pYZ47 and the intact *plsX* gene on the chromosome. All elements are indicated. The thick line represents the *E. coli* chromosome, and the thin line represents the plasmid. Half-arrows with numbers above or below represent the PCR primers used in the study (sequences are given in Materials and Methods). M13, M13 reverse-sequencing (−48) primer purchased from New England Biolabs.

We began with the genes of the 3′ end of the cluster and reported transcriptional analyses of the *fabD*, *fabG*, *acpP*, and *fabF* genes (36). We also addressed the physiological relevance of the multiple *acpP* transcripts with a powerful genetic approach, polar allele duplication (Fig. 1). This method allows blockage of chromosomal transcription from sequences upstream of a given promoter without disruption of either coding sequences or downstream transcription. By use of this method, we showed that only one of the two major promoters that transcribe *acpP* is required for expression of physiological levels of this protein (36). In the present study, polar allele duplication was used to test the possibility that the upstream genes of the *fab* cluster (*plsX-fabH-fabD-fabG*) are transcribed as an operon. We were unable to isolate polar allele duplications of the *fabD-fabG* segment by the methods used for *acpP*, suggesting that transcription from an upstream promoter might be necessary for growth. In order to conduct a positive test of this hypothesis, we cloned the *fab* gene cluster from *Salmonella typhimurium*, since the cluster from this closely related bacterium should provide functional copies of the proteins needed for the growth of *E. coli* without providing a target for recombination with the *E. coli* gene segments needed to direct polar allele duplication (24). We report that in a *plsX* polar allele duplication strain, the *S. typhimurium fab* cluster plasmid, pYZ53, was not required for cell growth, indicating that the *plsX* gene has its own promoter(s) and that only that promoter(s) is required for expression of the PlsX protein and perhaps downstream Fab enzymes at physiological levels. In contrast, we found that the viability of a *fabG* polar allele duplication strain depended on expression of the *S. typhimurium fabG* gene and hence that distal promoters were required in order to obtain physiological levels of FabG. Polar allele duplication strains affecting *fabD* were found to grow very slowly, indicating that only a weak *fabD* promoter is present.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. All bacterial strains are derivatives of *E. coli* K-12 or *S. typhimurium* LT2. The *E. coli* strains and plasmids used in this study are listed in Table 1. *S. typhimurium* MST2370 contains a locked-in Mud-P22 at min 21.5 (*putA1019*::MudQ) of the *S. typhimurium* linkage map which packages in the clockwise direction (2). The Mud-P22 phage DNA isolated from *S. typhimurium* MST2370 after induction with mitomycin C (35) was digested to completion with either *Eco*RV or *Nru*I, and the fragments were ligated to pHSG575 cut with *Sma*I. The ligation products were transformed into an *E. coli fabD* mutant strain, LA2-89 (which is deficient in malonyl-CoA-ACP transacylase activity at 42°C), to select for complementing clones (13, 34). One each of the *Eco*RV- and *Nru*I-derived plasmids (called pYZ48 and pYZ58, respectively), was retained and again transformed into strain LA2-89 to confirm complementation. Strain YZ133, which harbored plasmid pYZ53, containing *S. typhimurium fab* cluster DNA, was transformed with plasmid pYZ47 to produce the *plsX* polar duplication strain YZ137. Strain YZ152, which harbored plasmid pYZ60, containing *S. typhimurium fab* cluster DNA, was transformed with either plasmid pYZ37 or plasmid pYZ69 to produce the *fabG* (strain YZ157) or the *fabD* (strain YZ167) polar duplication strain, respectively. Plasmids pYZ47, pYZ37, and pYZ69 replicate from an R6Kγ replication origin and thus require the plasmid R6K-encoded Pir protein for replication. The wild-type *E. coli* recipient strains lack Pir, and thus transformants with plasmid-encoded antibiotic resistance (to ampicillin and chloramphenicol) result from integration of the plasmid DNA into the *E. coli* chromosome via homologous recombination between the 5′ portions of the *fabG*, *plsX*, or *fabD* genes. These recombinant strains were called YZ157, YZ137, and YZ167, respectively. The *recA* derivatives of strains YZ157, YZ137, and YZ167, called strains YZ158, YZ141 and YZ168, respectively, were constructed by transduction with a P1 phage lysate grown on strain JC10289 with selection for tetracycline resistance, followed by screening for UV sensitivity. Derivatives of strain YZ141 that were kanamycin sensitive (indicating loss of the *S. typhimurium fab* cluster plasmid pYZ53) were obtained by screening colonies that arose after cells were plated on rich broth (RB) agar plates lacking kanamycin. Strains YZ158 and YZ168 were cured of plasmid pYZ60 by transformation with the incompatible plasmids pYZ71 and pYZ72 and were then screened for colonies that were resistant to spectinomycin and sensitive to kanamycin to produce strains YZ166 and YZ170, respectively.

Culture media and growth conditions. Minimal E medium supplemented with 4,000 mg of glucose/liter, 100 mg of methionine/liter, and 10 mg of thiamine/liter or RB was used for growth of bacterial strains (16). Antibiotics were added at the following concentrations (in milligrams per liter): kanamycin, 25; ampicillin, 100; tetracycline, 30; and chloramphenicol, 34. Bacterial growth was monitored with a Klett-Summerson colorimeter with a green filter.

Plasmid isolation and recombinant DNA techniques. Plasmid isolation was performed by either a modified alkaline lysis method (11) or Qiagen Spin minipreparations. Southern blot analyses were carried out according to the Genius System User's Guide (Boehringer Mannheim Biochemicals). The probes were plasmid pYZ37, pYZ47, and pYZ69 labeled with digoxigenin (DIG)-dUTP via random-primed labeling with the Genius 2 DNA labeling kit, purchased from Boehringer Mannheim. *S. typhimurium* Mud-P22 phage lysate preparation and DNA isolation were performed according to the procedure of Youderian and coworkers (35). Low-stringency Southern blot analysis was performed with a DIG-dUTP-labeled PCR fragment (amplified with primers 10 and 13) that contained the *E. coli fabD* and *fabG* genes, plus a 5′ fragment of *acpP*. Other DNA manipulations were performed by standard procedures (26).

DNA sequencing of both strands of the *S. typhimurium fab* cluster genes on plasmid pYZ48 and pYZ58 was done by the Genetic Engineering Facility, University of Illinois at Urbana-Champaign, with *Taq* DNA polymerase cycle sequencing on an Applied Biosystems 373 DNA sequencer, with primers designed and synthesized by the facility staff.

RNA analyses. Total RNA was isolated from exponentially growing cells by the rapid isolation method (1). Reverse transcriptase-coupled PCR (RT-PCR) was performed with the RETROscript kit, purchased from Ambion. The primer used for the first cDNA strand synthesis was the random decamer mixture provided in the kit. The primers used in PCR are listed below. Quantitative RT-PCR was carried out with the Ambion kit according to the protocol of Gilliland and coworkers (6). Briefly, plasmids pYZ64 and pYZ66 were used as templates with primers 1 and 7 and primers 6 and 5, respectively, to amplify the competitive DNA fragments. The concentrations of the competitive DNA fragments were then determined either by absorption at 260 nm or by comparing the fluorescence intensities with those of a DNA mass ladder (purchased from Gibco BRL) by densitometry of ethidium bromide-stained agarose gels. The reverse transcriptase (RT) reaction product (1 μl) and different concentrations of competitive DNA (as specified in the legend to Fig. 4) were added to a 25-μl PCR mixture. The same sets of primers used to amplify the competitive DNA were used in the RT-PCRs. The PCR products were separated on an agarose gel stained with ethidium bromide and quantitated by densitometry. The ratios of the fluorescence intensities of the PCR products of the competitive DNA to those of the RT-PCR products were plotted as a function of the concentration of the competitive DNA (6).

For Northern blot analysis, whole-cell lysates were separated by electrophoresis on 0.8% formaldehyde agarose gels as described by Kornblum et al. (9). Northern transfer was performed by standard procedures (26). Hybridization, washing, and detection were carried out as described by the Genius System User's Guide. Other experimental conditions are given in the legend to Fig. 6.

TABLE 1. Plasmids and *E. coli* strains used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
UB1005	F⁻ *metB1 relA1 spoT1 gyrA216* λ^r/λ$^-$	Lab collection
LA2-89	*fabD*(Am) *supE1* [*fabD*(Ts) phenotype]	Lab collection (31)
WM95	F′ 128::Tn*10-11 lacI*^{q} Δ*lacZ*M15/*l*DE3 Δ*lacX74 uidA*::*pir recA1 rpsL*	W. W. Metcalf (15)
JC10289	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 thi-1* Δ(*recA-srl*)*306 rpsL31 srlR301*::Tn*10-84*	CGSC[a]
YZ125	LA2-89/pYZ48	This work
YZ133	UB1005/pYZ53	This work
YZ137	YZ133::pYZ47 (*plsX* polar duplication on chromosome)	This work
YZ141	YZ137 Δ(*recA-srl*)*306 srlR301*::Tn*10-84*	This work
YZ142	LA2-89/pYZ58	This work
YZ143	Kan^s derivative of strain YZ141 (loss of pYZ53)	This work
YZ152	UB1005/pYZ60	This work
YZ157	YZ152::pYZ37 (*fabG* polar duplication on chromosome)	This work
YZ158	YZ157 Δ(*recA-srl*)*306 srlR301*::Tn*10-84*	This work
YZ159	UB1005 Δ(*recA-srl*)*306 srlR301*::Tn*10-84*	This work
YZ166	Transformation of pYZ71 into strain YZ158 to cure pYZ60	This work
YZ167	YZ152::pYZ69 (*fabD* polar duplication on chromosome)	This work
YZ168	YZ167 Δ(*recA-srl*)*306 srlR301*::Tn*10-84*	This work
YZ170	Transformation of pYZ72 into strain YZ168 to cure pYZ60	This work
Plasmids		
pACYC177	Ap^r Kn^r cloning vector; contains p15A replicon	Lab collection
pHSG575	Cm^r cloning vector; contains pSC101 replicon	27
pWM77	Suicide vector derived from pJM703.1	W. W. Metcalf (15)
pYZ37	Insertion of the 700-bp *Pst*I-*Eco*RV fragment (*fabG′*) of pKM22 into pWM77 with the 3.5-kb Ω-Cm fragment of pHP45Ω-Cm immediately upstream in the *Bam*HI site	This work
pYZ46	500-bp *plsX′* PCR product of the *E. coli* chromosome (amplified with primers 8 and 3) treated with T4 DNA Polymerase, then cut with *Sal*I and inserted into pWM77 cut with *Sma*I and *Sal*I	This work
pYZ47	Insertion of the 3.5-kb Ω-Cm *Bam*HI fragment of pHP45Ω-Cm into the *Bam*HI site of pYZ46	This work
pYZ48	Insertion of the 3.8-kb *Eco*RV *fab* gene fragment of Mud-P22 phage DNA from *S. typhimurium* MST2370 into the *Sma*I site of pHSG575	This work
pYZ53	Insertion of the 3.8-kb *Bam*HI-*Eco*RI fragment of pYZ48 into pACYC177 cut with *Bam*HI and *Dra*I	This work
pYZ58	Insertion of the 2.2-kb *Nru*I *fab* gene fragment of Mud-P22 phage DNA from *S. typhimurium* MST2370 into the *Sma*I site of pHSG575	This work
pYZ59	Insertion of the *Bam*HI-*Pvu*II fragment of pYZ48 and the *Pvu*II-*Eco*RI fragment of pYZ58 together into pHSG575 cut with *Bam*HI and *Eco*RI	This work
pYZ60	Insertion of the 4.6-kb *Bam*HI-*Eco*RI (filled-in) fragment of pYZ59 into pACYA177 cut with *Bam*HI (filled in) and *Dra*I	This work
pYZ63	Insertion of the 652-bp PCR product of *E. coli* chromosomal DNA (amplified with primers 1 and 3) into pUC19 cut with *Bam*HI and *Hin*dIII (filled in)	This work
pYZ64	Plasmid pYZ63 was cut with *Hin*dIII and *Pst*I and then blunt ended with T4 DNA polymerase and ligated	This work
pYZ65	Insertion of the 459-bp PCR product of the *E. coli* chromosome (amplified with primers 2 and 5) into pUC19 cut with *Bam*HI and *Hin*dIII (filled in)	This work
pYZ66	Plasmid pYZ65 was cut with *Pst*I and then ligated	This work
pYZ67	Insertion of the 600-bp *fabD′* PCR product of *E. coli* chromosomal DNA (amplified with primers 9 and 11) cut with *Eco*RI and *Hin*dIII into pBluescript II SK cut with *Eco*RI and *Hin*dIII	This work
pYZ68	Insertion of the 3.5-kb Ω-Cm *Bam*HI fragment of pHP45Ω-Cm into the *Bam*HI site of pYZ67	This work
pYZ69	Insertion of the 4.2-kb *Sal*I-*Not*I fragment of pYZ68 into pWM77 cut with *Sal*I and *Not*I	This work
pYZ70	Recircularization of the 4.5-kb *Xho*I fragment of pMPM-K6Ω (deletion of the Kan^r gene)	This work and reference 14
pYZ71	Insertion of the 770-bp PCR product of pYZ58 (amplified with primers 16 and 17) into pYZ70 cut with *Nco*I and *Xmn*I to construct a gene fusion with *S. typhimurium fabG* under the control of the arabinose promoter	This work
pYZ72	Insertion of the 1,720-bp PCR product of pYZ58 (amplified with primers 18 and 17) into pYZ70 cut with *Nco*I and *Xmn*I to construct a gene fusion with *S. typhimurium fabD* and *fabG* under the control of the arabinose promoter	This work

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Primers used in RT-PCR and other manipulations. In addition to the M13 16-mer reverse-sequencing primer from New England Biolabs, the primers used (sequences shown 5′ to 3′) were as follows: primer 1, GCAATGGTTGAAGA TGAAATCATCC; primer 2, GTTAGATCATATGGGAGGG; primer 3, GAC GTCGACGTTGTTCAAAGTCAG; primer 4, CTGACTGCGCAGGAATAAT CTGC; primer 5, CACCAGCATTGTGCTGTCACAACT; primer 6, GTTAG ATGTCATGGGAGGGGATTT; primer 7, GACGCGAACGTTGTTCAAAG TCAG; primer 8, TCGTTGGATCGGGGATAAACCG; primer 9, CTGGCGC

GCACCTGCGATCCAA; primer 10, GGGAATTCTTGACCGTTCTCAACT GG; primer 11, CGCAACAGATGCAGTCAACAG; primer 12, GCGAATTC GAAACCAATGGTGATGC; primer 13, GGTCTTCAACCTAAGAAGCATT GTTGG; primer 14, GAAGTTACCAACAATGCTTC; primer 15, TCCTGAT CAGACACGTTTGTCCTCCAGGGA; primer 16, GGAAAATCATGAGCTT TGAAGG; primer 17, CCCTAATAACGCAAATATTTTTC; and primer 18, GGATTTAATCATGACGCAA.

Genetic techniques. Transduction was carried out according to the method of Miller (16). Allele duplication was done as described by Metcalf et al. (15). Plasmids pYZ37, pYZ47, and pYZ69 (Table 1), which contain the 5′ portions of the *fabG*, *plsX*, and *fabD* genes, respectively, were maintained in the Pir-containing strain, WM95, and were then transformed into the wild-type strains YZ152, YZ133, and YZ152, respectively (which lack Pir), followed by selection for transformants resistant to both ampicillin and chloramphenicol.

Nucleotide sequence accession number. The nucleotide sequence of the *S. typhimurium fab* gene cluster has been submitted to GenBank under accession no. AF044668.

RESULTS

Cloning and sequencing of the *S. typhimurium fab* cluster. As will be described below, we had failed to isolate various polar allele duplications within the *E. coli fab* cluster. This could be due to poor luck (successful transformations give only 10 to 20 colonies) or to disruption of essential transcription. In order to cope with the latter possibility, we cloned the *fab* cluster from *S. typhimurium*, a close relative of *E. coli*, and used plasmids carrying this DNA fragment to provide any essential proteins lost due to polar allele duplication. Our isolation of the *S. typhimurium fab* cluster was based on two assumptions: (i) that the overall organization of the *S. typhimurium fab* cluster would closely resemble that of *E. coli* (22), since the *fab* gene cluster is widely conserved among much more distantly related bacteria (5, 17, 27, 28) and (ii) that, given the similarities of the genetic maps of the two bacteria, the *fab* gene cluster of *S. typhimurium* would be located at about genome min 24. To test if these assumptions were correct, we used a "locked-in" Mu*d*-P22 prophage (*putA1019*::Mu*dQ*) integrated at min 21.5 of the *S. typhimurium* genetic map (2, 35). Upon induction of this phage with mitomycin C, it cannot escape from the bacterial chromosome, and it packages successive phage headfuls of *S. typhimurium* chromosomal DNA in a clockwise direction (2). The phage particles in the lysate were isolated, and the encapsidated DNA was purified and digested with various restriction enzymes. Low-stringency Southern blot analysis was performed with a DIG-dUTP-labeled fragment (obtained by PCR with primers 10 and 13) which contains the complete *E. coli fabD* and *fabG* genes plus a 5′ fragment of the *acpP* gene. Positive bands were detected (data not shown), suggesting that the locked-in phage DNA did indeed contain the *S. typhimurium fab* cluster genes.

We cloned the *S. typhimurium fab* cluster genes from the phage particle DNA by complementation of an *E. coli* mutant deficient in malonyl-CoA-ACP transacylase activity at 42°C. Strain LA2-89 carries an amber mutation in the *fabD* gene together with a *supE* tRNA suppressor (34). The combination of these two characteristics results in both a temperature-sensitive malonyl-CoA-ACP transacylase and temperature-sensitive growth. The phage particle DNA was digested to completion with each of a variety of different restriction enzymes, and the fragments were then ligated to the low-copy-number vector pHSG575 (29). The resulting plasmids were transformed into a restriction-deficient *E. coli* strain, and plasmid preparations from pools of the resulting transformants were used to transform strain LA2-89, followed by selection for chloramphenicol-resistant clones that grew at 42°C. Only the plasmid pool constructed from *Eco*RV fragments gave transformants. One of these isolates, pYZ48, was sequenced and was found to contain homologs of *E. coli rpmF*, *plsX*, *fabH*, and *fabD*, plus two partial gene fragments, the 5′ end of *fabG* and the 3′ end of *g30k*, an open reading frame (ORF) of unknown function located upstream of *rpmF*. In order to obtain the remainder of the *fabG* gene, the phage particle DNA was digested with *Nru*I, which cuts only once in the pYZ48 insert DNA (within the *fabH* gene), and was ligated to pHSG575 cut with *Sma*I, and plasmids that complemented *E. coli* LA2-89 were again selected. One such clone, pYZ58, was retained, sequenced, and used to construct a plasmid that carried a cluster with an intact *fabG* gene.

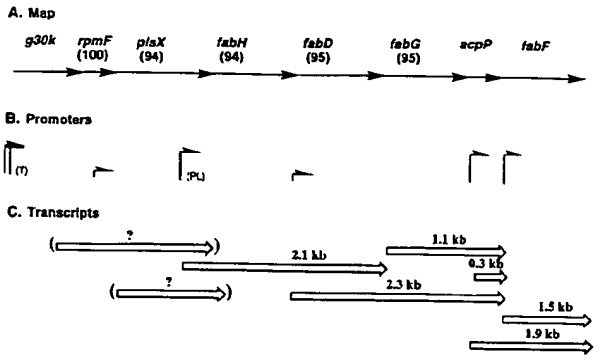

FIG. 2. Organization of the *fab* cluster genes, their promoters, and transcripts. (A) The order of the genes in *E. coli* and *S. typhimurium* is identical. The percentages of amino acid residues identical in the *S. typhimurium* and *E. coli fab* cluster proteins are given above the arrows representing the individual genes. The *S. typhimurium* nucleotide sequence is about 85% identical to the *E. coli fab* gene cluster sequences. (B) The known promoters of the region are depicted (the heights of the arrows are intended to give a crude idea of relative promoter strengths). The promoters upstream of *g30K* (marked T) are those identified by S1 nuclease mapping by Tanaka and coworkers (30). The promoter within *plsX* (PL) is that identified by primer extension studies by Podkovyrov and Larson (21). (C) The transcripts identified in this study and our prior studies (36) are shown. The *plsX* transcripts were detected only by RT-PCR, and thus the lengths of these transcripts are unknown. The scarcity of the longer *plsX* transcript could be due to termination of most of the transcripts initiated at the promoters upstream of *g30K* at the terminator mapped downstream of *rpmF* (30).

The deduced protein product of each *S. typhimurium* gene has the same number of residues as the *E. coli* homolog, except that the *plsX* ORF product is 3 residues longer than its *E. coli* homolog. Each of the deduced proteins has >90% amino acid identity to the analogous *E. coli* protein (Fig. 2), and thus the nomenclature of the *S. typhimurium fab* genes is the same as that of the *E. coli* genes. The only noteworthy difference between the *fab* gene clusters of the two organisms was a 55-bp deletion within the *S. typhimurium fabG-acpP* intergenic region compared to that of *E. coli* (the intergenic regions between other *S. typhimurium fab* cluster genes were very similar to those of *E. coli*).

Construction of a *plsX* gene polar allele duplication. We used polar allele duplication (Fig. 1) to demonstrate that the promoter located immediately upstream of the *acpP* gene is sufficient for expression of ACP at physiological levels (36). In the present study, we extended this approach to the upstream genes of the *E. coli fab* cluster. A PCR product beginning 40 bp upstream of the small *rpmF* coding sequence and ending 150 bp within the *plsX* coding sequence was inserted into the oriR6Kγ plasmid, pWM77 (15), immediately downstream of an Ω-Cm cassette that blocks transcription from upstream genes. We transformed the resulting plasmid, pYZ47, into the wild-type *E. coli* strain UB1005, which lacks Pir (and is therefore unable to replicate pYZ47), and transformants resistant to both chloramphenicol and ampicillin were selected. Such transformants can be formed only by single crossover of the

plasmid into the chromosome (Fig. 1). Several failed attempts to construct this strain, together with the data of Podkovyrov and Larson (20), suggested that cotranscription of the *plsX* gene and downstream *fab* genes with the upstream ribosomal protein gene, *rpmF*, might be required for growth.

To test this possibility, plasmid pYZ53, containing the complete *S. typhimurium rpmF*, *plsX*, *fabH*, and *fabD* genes, plus the 5′ end of the *S. typhimurium fabG* gene (the insert DNA is the same as that of plasmid pYZ48), was transformed into the wild-type strain UB1005 to produce strain YZ133. This strain was then transformed with pYZ47 to obtain the polar allele duplication. The rationale was that the *S. typhimurium fab* genes would provide any *E. coli* chromosomal functions lost as a result of the formation of the polar allele duplication but would not be a substrate for recombination with the *E. coli* sequences of plasmid pYZ47. Colonies resistant to kanamycin (indicating the presence of the *S. typhimurium fab* cluster plasmid pYZ53), chloramphenicol, and ampicillin (Fig. 1) were obtained. PCR analyses using primer 1 and the M13 reverse-sequencing primer (Fig. 1), plus Southern analysis of one of such recombinant strain, YZ137, verified the expected integration of plasmid pYZ47 into the *E. coli* chromosome (data not shown). A *recA* mutation was then transduced into the strain to produce the stabilized strain YZ141.

Strain YZ141 was grown on RB agar lacking kanamycin (in agreement with the observations of prior workers [23], it was found that p15A origin plasmids were not stably maintained without selection) to test if pYZ53 (which contains the *S. typhimurium fab* genes) is essential for cell viability. About 300 colonies were screened for kanamycin resistance, and about 20% of the colonies were kanamycin sensitive. One of these kanamycin-sensitive derivatives (called YZ143) was further tested by plasmid isolation and Southern analysis to confirm both the loss of *S. typhimurium fab* gene-containing plasmid pYZ53 and the presence of the expected *plsX* polar allele duplication on the *E. coli* chromosome (data not shown). This strain had the expected chromosomal map, indicating that a promoter(s) located upstream of the *plsX* gene provides sufficient transcription to support cell viability. This promoter probably lies within the *rpmF* gene, based on the studies of Podkovyrov and Larson (20), whereas the longer *plsX* transcript probably originates at the two promoters mapped upstream of *g30k* by Tanaka and coworkers (30).

Transcription of the *plsX* gene. RT-PCR was used to detect and quantitate *plsX* transcription in strains UB1005 and YZ143, since several attempts to perform Northern analysis of *plsX* transcription failed due to the scarcity of the transcripts. When the primer pair 1 and 3 (see Fig. 1) was used, both strain UB1005 and strain YZ143 gave RT-PCR products of 652 bp (Fig. 3, lanes 4 and 5), consistent with cotranscription of the *plsX* gene with the upstream ribosomal protein gene, as suggested by Podkovyrov and Larson (20). When primers 1 and 4 (Fig. 1) were used to prime RT-PCR, a product of the expected length was detected in strain UB1005 (Fig. 3, lane 2) but not in strain YZ143 (Fig. 3, lane 3), demonstrating that the polar allele duplication indeed blocked transcription from upstream. These total-RNA preparations were also tested in direct PCRs (in the absence of RT) with the same sets of primers (Fig. 3, lanes 7 to 10) to rule out the possibility of DNA contamination of the RNA preparations. Primers 2 and 5 (Fig. 1) were also used in RT-PCR analysis, and products of the expected length were detected both in strain UB1005 and in strain YZ143 (Fig. 4 and data not shown), a result consistent with the viability of strain YZ143.

Quantitative RT-PCR (6) was used to assess the ratio of the level of the transcript containing both *plsX* and *rpmF* to that of the transcript containing only *plsX*. The principle of this method is to utilize a known concentration of a DNA fragment (obtained by amplification with the same primers used in the RT-PCRs) to compete with the RT-PCR product. To obtain the needed competitive DNA fragments, 652- and 459-bp PCR fragments were amplified from *E. coli* chromosomal DNA with primer pair 1 and 3 and primer pair 2 and 5, respectively, and the amplified fragments were cloned into vector pUC19. A *Hin*dIII-*Pst*I fragment and a *Pst*I fragment, respectively, were deleted from the plasmid inserts to produce plasmids pYZ64 and pYZ66, respectively. These plasmids generate amplification products of 522 and 346 bp, respectively, as competitive DNA fragments. Two primers, 6 and 7, were paired with primers 5 and 1, respectively; primer pair 6 and 5 and primer pair 1 and 7 were used to amplify competitive DNA fragments from plasmids pYZ66 and pYZ64, respectively, and the concentrations of the competitive DNA fragment solutions were determined as described in Materials and Methods. RT-PCR was carried out with known concentrations of competitive DNA added to the reaction mixtures. A total-RNA preparation from strain UB1005 was used as the RT template, and the concentration of cDNA formed was taken to be proportional to the mRNA concentration. Primer 1 anneals to a sequence in *g30k* upstream of *rmpF*, and primer 6 anneals to a sequence at the 5′ end of the *plsX* gene, whereas both primer 5 and primer 7 anneal to sequences in the center of the *plsX* gene (Fig. 1). Therefore, the RT-PCR product from primer pair 1 and 7 represents only the products of cotranscription of *rmpF* and *plsX*, whereas the product from primer pair 6 and 5 represents the total of the *plsX* transcripts. When decreasing concentrations of the 522-bp competitive DNA fragment synthesized with primers 1 and 7 were added to the reaction mixtures for RT-PCR of strain UB1005 with the same primer pair, it was shown that the concentration of the 652-bp RT-PCR product increased while the concentration of the 522-bp PCR product from the competitive DNA template decreased (Fig. 4A, lanes 1 to 8). The ratios of the fluorescence intensities of the PCR

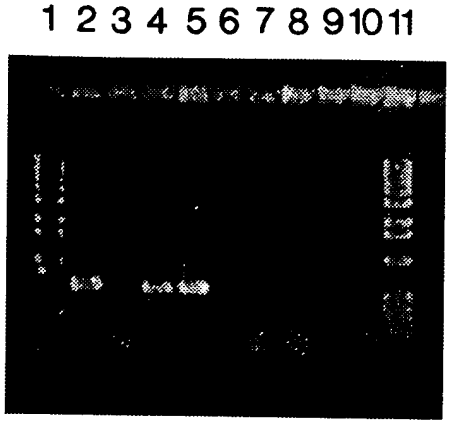

FIG. 3. RT-PCR analysis of *plsX* gene expression in the *plsX* polar allele duplication strain. The *E. coli plsX* polar allele duplication strain YZ143 and the wild-type strain UB1005 were grown to mid-log phase in RB, and total RNA was prepared as described in Materials and Methods. RT-PCRs were performed by using the Ambion RETROscript kit as directed. Lanes 2 through 5, RT-PCRs; lanes 7 through 10, PCRs with approximately 0.5 μg of total RNA as the template, but lacking RT. One microliter of RT reaction product, which corresponded to approximately 0.5 μg of total RNA, was used for each RT-PCR. The primer pairs used were 1 and 4 (lanes 2, 3, 7, and 8) or 1 and 3 (lanes 4, 5, 9, and 10). In lanes 2, 4, 7, and 9, RNA from strain UB1005 was analyzed. In lanes 3, 5, 8, and 10, RNA from strain YZ143 was analyzed. Lanes 1 and 11 are the 1-kb DNA ladder from BRL, and lane 6 was left vacant.

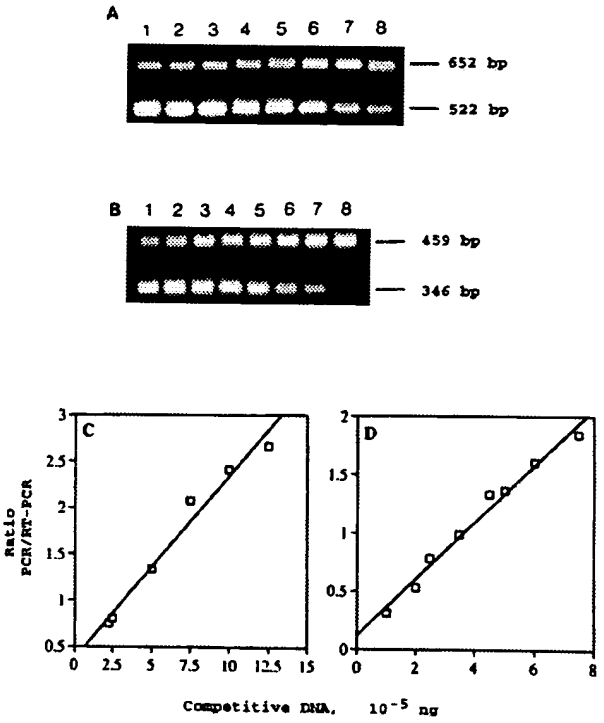

FIG. 4. Analysis of *plsX* gene expression by quantitative RT-PCR. RT-PCRs were performed as described for Fig. 3, except that different masses of competitive DNA were added to a given mass of RT-PCR mixture. (A) Quantitative RT-PCR with primers 1 and 7. Different masses (15×10^{-5}, 12.5×10^{-5}, 10×10^{-5}, 7.5×10^{-5}, 5×10^{-5}, 2.5×10^{-5}, or 2.25×10^{-5} ng) of competitive DNA amplified from pYZ64 with primers 1 and 7 were added to the RT-PCR mixtures in lanes 1 through 8, respectively. (B) Quantitative RT-PCR with primers 6 and 5. Competitive DNA (7.5×10^{-5}, 6.5×10^{-5}, 4.5×10^{-5}, 3.5×10^{-5}, 2.5×10^{-5}, 2×10^{-5}, or 1×10^{-5} ng) was added to lanes 1 through 8, respectively. The fluorescence intensities of the bands on the agarose gel in each lane were quantified with densitometry following ethidium bromide staining. The ratio of the intensity of the PCR product of competitive DNA to that of the RT-PCR product was calculated for each reaction, and these ratios were plotted as a function of the competitive DNA concentration. (C and D) Plots of data from panels A and B, respectively.

products to those of the RT-PCR products were plotted as a function of the concentration of the competitive DNA (Fig. 4C). (When the ratio is 1, the molar concentration of the competitive DNA added to the reaction mixture is identical to the molar concentration of the *plsX* cDNA, which is proportional to the *plsX* mRNA concentration). The level of cDNA synthesized from the *rpmF-plsX* cotranscripts was 6.40×10^{-5} ng/mg of UB1005 total RNA (Fig. 4C). Likewise, when the 346-bp competitive DNA fragment obtained with primers 6 and 5 from pYZ66 was added to the RT-PCR mixture (Fig. 4B and D), the concentration of cDNA synthesized by using the *plsX* total transcripts as the original template was 7.23×10^{-5} ng/mg of UB1005 total RNA. Therefore, when converted to molar quantities, these RT-PCR data indicate that about 60% of *plsX* transcription initiated at the promoters mapped upstream of the *g30K* gene by Tanaka and coworkers (30), whereas only 40% originated from the *plsX*-specific promoter (mean of three experiments).

The normal growth rate of the *plsX* polar allele duplication strain YZ143 (Fig. 5) indicated that transcription from the promoter(s) upstream of *rpmF* was not required for growth.

Construction of *fabG* and *fabD* polar allele duplications. Initial attempts to construct polar allele duplications upstream

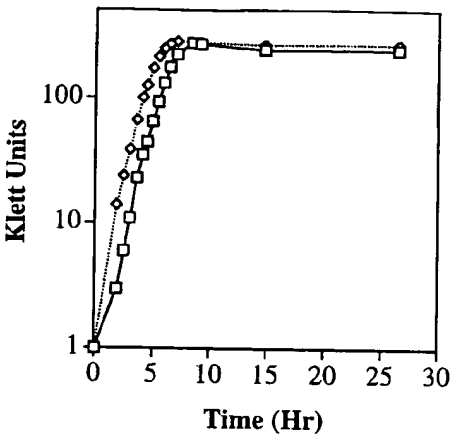

FIG. 5. Growth curve of the *plsX* polar allele duplication strain YZ143. Strain YZ143 (□) and strain YZ159 (a *recA* derivative of strain UB1005) (◇) were grown in the supplemented minimal E medium described in Materials and Methods. Growth was monitored with a Klett-Summerson colorimeter with a green filter.

of *fabG* or *fabD* were unsuccessful, and to avoid the possibility of disrupting essential transcription, we constructed a plasmid that contained intact copies of the *S. typhimurium rpmF*, *plsX*, *fabH*, *fabD*, and *fabG* genes. This construct was assembled in vector pHSG575 by a tripartite ligation using the inserts of plasmid pYZ48 and pYZ58 and then was subcloned into the kanamycin-resistant p15A vector, pACYC177. The resulting plasmid, pYZ60, was transformed into the wild-type strain UB1005 to give strain YZ152. Strain YZ152 was then transformed with plasmid pYZ37, which contains a 240-bp segment of the *fabG* coding sequence plus 300 bp of upstream sequence (a chromosomal *Pst*I-*Eco*RV fragment) with an Ω-Cm cassette upstream of *fabG'*, or with plasmid pYZ69, which contains a 90-bp segment of the *fabG* coding sequence plus 470 bp of upstream sequence (a chromosomal *Eco*RI-*Hin*dIII fragment) with an Ω-Cm cassette inserted upstream of *fabD'*, and transformants resistant to both chloramphenicol and ampicillin were selected. The expected integration events were confirmed by PCR with the M13 reverse-sequencing primer plus either primer 10 (for *fabG*) or primer 6 (for *fabD*) and by Southern analysis as described above (data not shown). Strains YZ157 (*fabG* duplication) and YZ167 (*fabD* duplication) were then stabilized by introduction of a *recA* mutation to produce strains YZ158 and YZ168, respectively.

Transcription of the *fabD* and *fabG* genes in the polar allele duplication strains. Strain YZ158, containing a *fabG* polar allele duplication, and strain YZ168, containing a *fabD* polar allele duplication, were first tested to see if plasmid pYZ60, which carries the *S. typhimurium fab* gene cluster, was required for growth. We first grew the strains without kanamycin selection in liquid medium and then screened for kanamycin-sensitive colonies without success (300 colonies of each strain were screened). We then cloned the *S. typhimurium fabG* gene into pYZ70, a kanamycin-sensitive derivative of vector pMPM-K6Ω (14) in which the intact gene was positioned such that it was transcribed exclusively from the vector arabinose-regulated *araBAD* promoter and was translated by using the vector ribosome binding site. The resulting spectinomycin-resistant plasmid, pYZ71, was used to transform strain YZ158, with selection for transformants resistant to ampicillin, chloramphenicol, tetracycline, and spectinomycin, followed by screen-

ing for kanamycin sensitivity. All these steps were carried out in RB medium supplemented with 0.2% arabinose to induce expression of *S. typhimurium fabG*. Since plasmids pYZ60 and pYZ71 share the p15A replication origin, plasmid incompatibility due to the presence of pYZ71 was expected to cure this strain of pYZ60. One such cured strain (YZ166) resistant to ampicillin, chloramphenicol, tetracycline, and spectinomycin but sensitive to kanamycin was retained, and the presence of a single plasmid, pYZ71, was confirmed by plasmid isolation and restriction analysis (data not shown).

Strain YZ166 was first assayed for dependence on arabinose-induced *S. typhimurium fabG* gene expression by streaking the strain on media supplemented with either 0.2% arabinose to induce the arabinose promoter or 0.4% glucose plus 0.002% fucose to decrease basal expression of the arabinose promoter (18). We found that strain YZ166 gave no detectable growth on plates supplemented with glucose-fucose, whereas it grew as well as the wild-type strain, UB1005, on plates supplemented with arabinose. Moreover, strain YZ166 failed to grow in the absence of arabinose, indicating that basal expression from the *araBAD* promoter was unable to support growth. Therefore, blocking of the transcription of *fabG* from upstream promoters was lethal to the cell unless another source of FabG was supplied.

Northern analysis was also performed to examine *fabG* transcription in strain YZ166. We did not use a *fabG*-specific probe because it could be difficult to distinguish transcription of the *E. coli* chromosomal *fabG* gene from that of the plasmid-borne *S. typhimurium fabG* gene. Instead, we used a probe that contained only the 3′ end of the *acpP* gene. We previously found (36) that *fabG* and *acpP* are cotranscribed to give two products of 1.1 and 2.3 kb (see Fig. 6A, lanes 1 and 2). Both transcripts disappeared in the *acpP* polar duplication strain, although the other transcripts, of 0.3 and 1.9 kb, which initiate at the *acpP* promoter (36), were unaffected (Fig. 6A, lane 3). Essentially the same result was given upon insertion of the Ω-Cm cassette between the *fabD* and *fabG* genes (Fig. 6A, lane 4). Therefore, in contrast to our findings with *acpP* and *plsX*, the *fabG* gene lacks a promoter immediately upstream of its coding sequence that is sufficiently strong to provide sufficient levels of gene product to support growth. These results also indicate that the 1.1-kb mRNA is produced by processing of a longer transcript(s). This also is the first evidence demonstrating *fabG* to be an essential gene.

Similar constructions gave pYZ72, in which the *S. typhimurium fabD* and *fabG* genes were placed under the control of the *araBAD* promoter. This plasmid was used to transform strain YZ168, and colonies resistant to ampicillin, chloramphenicol, tetracycline, and spectinomycin were selected and then screened for kanamycin sensitivity in the presence of arabinose as described above. The resulting strain, YZ170, was tested for the presence of plasmids, and only plasmid pYZ72 was detected. Strain YZ170 was assayed for dependence on the *S. typhimurium fabD fabG* genes carried by plasmid pYZ72 as described above. To our surprise, we found that there was detectable growth of strain YZ170 on the glucose-fucose medium, although the colonies were much smaller and grew less densely than those formed on medium supplemented with arabinose (data not shown). This slow growth seemed to be due to low-level expression of *fabD* (rather than of *fabD* plus *fabG* or of *fabG* alone), since substitution of pYZ71, the arabinose-regulated *fabG* plasmid, for the *fabD fabG* plasmid, pYZ72, produced only a very modest increase in growth upon the addition of arabinose, indicating that FabG levels did not limit growth under these conditions.

The slow growth of the *fabD* polar duplication strain indicated that a weak promoter(s) located immediately upstream of the *fabD* coding sequence transcribes *fabD* and *fabG*. The weak nature of this promoter(s) was evident in Northern analyses. The same *acpP*-specific probe used in the Northern analysis of *fabG* transcription (see above) was used to assay expression of the *fabD* gene in the *fabD* polar duplication strain, YZ170. In contrast to results with the *acpP* and *fabG* duplication strains, insertion of the Ω-Cm cassette between the *fabH* and *fabD* genes resulted in greatly decreased levels of the 1.1- and 2.3-kb transcripts (the 2.3-kb transcript was virtually undetectable) (Fig. 6B, lanes 4 and 5), a result consistent with the slow growth of strain YZ170. These results indicated that the promoter located immediately upstream of *fabD* is only weakly functional and that most of the 1.1-kb transcript was the processed product of a longer transcript(s) initiated at promoters well upstream of *fabD*.

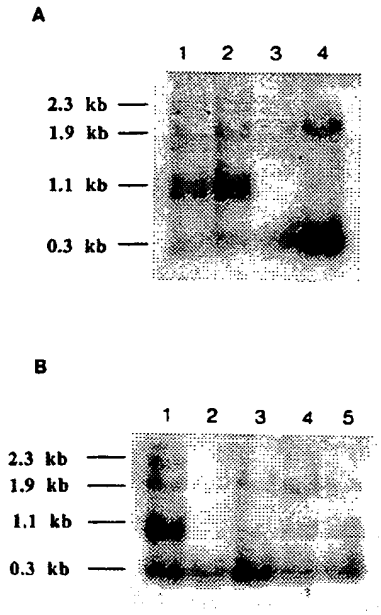

FIG. 6. Northern analysis of *fabG* and *fabD* gene expression in the *fabG* and *fabD* polar allele duplication strains YZ166 and YZ170. The strains were grown to mid-log phase in RB that was either unsupplemented (strains UB1005 and YZ60), supplemented with 0.2% arabinose (strain YZ166), or supplemented either with 0.2% arabinose or with 0.4% glucose plus 0.002% fucose (strain YZ170). Cell lysates were prepared as described in Materials and Methods. The probe used was the DIG-labeled PCR product from the 3′ end of *acpP* obtained by amplification with primers 14 and 15. (A) Northern analysis of *fabG* expression. Lanes 1 and 2, strain UB1005; lane 3, strain YZ60; lane 4, strain YZ166. (B) Northern analysis of *fabD* expression. Lane 1, UB1005; lane 2, strain YZ60; lane 3, strain YZ166; lane 4, strain YZ170 grown in RB supplemented with glucose plus fucose; lane 5, strain YZ170 grown in RB supplemented with arabinose.

DISCUSSION

We developed the polar duplication approach to overcome a deficiency of standard transcriptional mapping that arises when a gene is found to have multiple transcripts. The usual assumption is that protein production is a direct function of transcript abundance, and hence, major transcripts are considered more important than minor transcripts. However, this is not necessarily valid, since a minor transcript may be more efficiently translated than a major transcript, or a major transcript might be the processed product of a longer transcript, which consequently becomes scarce. On the other hand, a

minor transcript may reflect only the incomplete nature of most transcription terminators and hence may have no physiological importance. Another complication of standard transcriptional mapping is the processing of the primary products of transcription, which can be detected by the polar duplication method (see below). Use of the *S. typhimurium* homologs to provide possible essential functions during the construction of polar duplication strains increases the applicability of the approach. We chose *S. typhimurium* based on its close relatedness with *E. coli* (and hence their interchangeable gene expression signals) and the known lack of recombination between homologous genes in these organisms, due largely to mismatches at the third positions of codons and the resulting inhibition of recombination by mismatch repair (24). However, genes from other organisms, such as *H. influenzae*, could also be useful.

Our application of the polar duplication approach to the *fab* gene cluster shows that, although this gene cluster shows obligatory cotranscription of some pairs of genes, some genes have a promoter located immediately upstream of the coding sequence that provides sufficient transcription for normal growth. Examples of such genes are *acpP* and *plsX*. In the case of *plsX*, our data bear on the argument of Podkovyrov and Larson (20) that cotranscription of *rpmF* and *plsX* could play an important role in coordinating ribosome synthesis with cell membrane synthesis. If *rpmF-plsX* cotranscription is important, its lack might be expected to slow or block growth. However, this conclusion is tempered by the lack of information on *plsX* function. This gene was discovered by the ability of a mutant allele, *plsX50*, to allow effective supplementation of *plsB* mutants of *E. coli* with *sn*-glycerol 3-phosphate on certain carbon sources (10). Only one *plsX* allele has been characterized, and the interactions studies involved a single *plsB* allele. The *plsX50* mutation has been reported to be a single-base pair deletion upstream of the coding sequence (GenBank accession no. M96793), and it is unclear whether this mutation causes a gain of function or a loss of function relative to the wild-type gene, since no complementation studies have been reported. On the other hand, most bacterial genomes sequenced to date encode a PlsX homolog, and thus, this protein seems likely to play an important role in cellular physiology. The prevalence of *plsX*-like genes in bacterial genomes indicates that further study of this enigmatic *E. coli* gene is required.

In contrast to *plsX* and *acpP*, *fabG* lacks a proximal promoter. Insertion of the Ω-Cm cassette between *fabD* and *fabG* abolished the synthesis of both the 1.1-kb mRNA, a cotranscript of *fabG* and *acpP*, and the 2.3-kb mRNA, a transcript of *fabD*, *fabG*, and *acpP* (Fig. 6). These results indicate that the abundant 1.1-kb mRNA is not initiated from a promoter located immediately upstream of the *fabG* gene but is produced by processing of longer transcripts. This is also consistent with the fact that although the 1.1-kb mRNA is very abundant, a strong promoter could not be detected immediately upstream of the *fabG* coding sequence. Several DNA fragments containing the overlapping regions immediately upstream of the *fabG* coding sequence were cloned into a promoter detection vector in which the inserts can drive *lacZ* expression. None of the *fabG* fragments resulted in β-galactosidase levels significantly higher than background (data not shown). We conclude that cotranscription of *fabG* with upstream genes is required for growth.

Transcription of *fabD* provides a middle ground between the extremes of *acpP-plsX* and *fabG*. Our previous Northern analyses of *fabD* transcription were inconclusive. In repeated attempts, only faint and diffuse bands were detected with a *fabD* probe (36). Our present data show that although *fabD* retains a proximal promoter within 370 bp of its coding sequence, this promoter is not sufficiently strong to support normal growth, and therefore cotranscription of *fabD* with upstream genes is needed. These transcripts could initiate at the promoters located upstream of *g30k* (29) and/or at the promoter mapped within *plsX* in the primer extension studies of Podkovyrov and Larson (21). The presence of a weak *fabD* promoter is consistent with the data of Podkovyrov and Larson (20).

The *fabG* polar allele duplication strain carrying a plasmid with the *S. typhimurium fabG* gene under the control of the *araBAD* promoter also showed no detectable growth unless the *S. typhimurium fabG* was induced. These data indicate that the *fabG* gene is essential for growth, a conclusion that is of interest, since several ORFs in addition to *fabG* have been classified as β-ketoacyl-ACP reductases by various annotators of the *E. coli* genomic sequence. These classifications could be explained if β-ketoacyl-ACP reductases exist that were specific either for different acyl chain lengths or for synthesis of saturated versus unsaturated fatty acids existed. However, fractionation of *E. coli* cell extracts gave only a single enzymatic activity that functioned with all acyl chains tested (32), and purified FabG catalyzes all the β-ketoacyl-ACP reductions required in the de novo synthesis of the long-chain fatty acids of *E. coli* in a reconstituted in vitro system (8). For these reasons we doubt that these other ORFs play a role in membrane lipid synthesis; instead, we suggest that they function in reductions of β-ketoacyl-CoA intermediates in other pathways (e.g., poly-β-hydroxybutyrate synthesis). The fact that the *fabG* polar allele duplication strain YZ166 is a conditionally lethal mutant (the strain cannot grow in medium lacking arabinose) should allow the determination of the stage at which the fatty acid biosynthetic pathway is arrested upon depletion of FabG protein in strain YZ166.

It is not surprising that the *S. typhimurium* fatty acid biosynthetic gene cluster has very high sequence identity to *E. coli* homologs at both the nucleic acid and amino acid levels (Fig. 1). However, the 55-bp deletion within the intergenic region between the *fabG* and *acpP* genes of *S. typhimurium* relative to that of *E. coli* was unexpected, especially given that the other *fab* cluster intergenic regions are very similar in the two bacteria. The deletion removes 55 bp located upstream of a sequence which is identical to that of the *E. coli acpP* promoter we identified previously (36). Therefore, *S. typhimurium acpP* transcription may differ somewhat from that of *E. coli*.

Why are these *fab* genes clustered when some of the genes retain their own promoters? Since *E. coli* fatty acid synthesis is a very tightly coupled pathway in which only traces of intermediates are seen (12), it seems unlikely that there would be a need to alter the ratios of the proteins encoded by these genes. Internal promoters could provide the means to combat the natural polarity seen in operons and also to increase the expression of a noncatalytic protein like ACP, which is needed in large quantities. However, the effects of natural polarity can also be canceled by increasing the relative efficiencies of translation of downstream ORFs. It will be interesting to see if the *fab* clusters of other bacteria utilize the *E. coli* mix of multigenic and monogenic transcription.

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The *Pseudomonas aeruginosa rhlG* Gene Encodes an NADPH-Dependent β-Ketoacyl Reductase Which Is Specifically Involved in Rhamnolipid Synthesis

JESÚS CAMPOS-GARCÍA,[1] ALMA DELIA CARO,[1] REBECA NÁJERA,[1] RAINA M. MILLER-MAIER,[2] RAGHEB A. AL-TAHHAN,[2] AND GLORIA SOBERÓN-CHÁVEZ[1]*

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62250, México,[1] and Department of Soil and Water Science, University of Arizona, Tucson, Arizona 85721[2]

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A *Pseudomonas aeruginosa* gene homologous to the *fabG* gene, which encodes the NADPH-dependent β-ketoacyl-acyl carrier protein (ACP) reductase required for fatty acid synthesis, was identified. The insertional mutation of this *fabG* homolog (herein called *rhlG*) produced no apparent effect on the growth rate and total lipid content of *P. aeruginosa* cells, but the production of rhamnolipids was completely abrogated. These results suggest that the synthetic pathway for the fatty acid moiety of rhamnolipids is separate from the general fatty acid synthetic pathway, starting with a specific ketoacyl reduction step catalyzed by the RhlG protein. In addition, the synthesis of poly-β-hydroxyalkanoate (PHA) is delayed in this mutant, suggesting that RhlG participates in PHA synthesis, although it is not the only reductase involved in this pathway. Traits regulated by the quorum-sensing response, other than rhamnolipid production, including production of proteases, pyocyanine, and the autoinducer butanoyl-homoserine lactone (PAI-2), were not affected by the *rhlG* mutation. We conclude that the *P. aeruginosa rhlG* gene encodes an NADPH-dependent β-ketoacyl reductase absolutely required for the synthesis of the β-hydroxy acid moiety of rhamnolipids and that it has a minor role in PHA production. Expression of *rhlG* mRNA under different culture conditions is consistent with this conclusion.

Pseudomonas aeruginosa is a bacterium that can be isolated from many different habitats, including water, soil, and plants (5). *P. aeruginosa* is also an opportunistic human pathogen that causes serious nosocomial infections (8). The secretion of numerous toxic compounds and hydrolytic enzymes has been correlated with its pathogenicity (19). These exoproducts include different proteases, such as elastase, LasA protease, and alkaline protease, as well as phospholipase C, exotoxin A, pyocyanine, and rhamnolipids. The production of these compounds is considered to be a virulence-associated trait and is coordinately regulated by a mechanism called "quorum sensing" (11), which depends on the production of N-acylated homoserine lactones harboring acyl substituents of two different lengths; PAI-1 contains a 12-carbon chain (22), while PAI-2 contains a butanoyl moiety (23). These small diffusible signaling molecules activate gene expression at high bacterial densities through interaction with specific transcriptional activators, LasR (22) and RhlR (20), respectively.

The role of these exoproducts in soil or aquatic habitats has not been determined, but it is clear that environmental and clinical *P. aeruginosa* isolates do not represent different populations, since it has been shown that there is a major clone common to pathogenic and environmental isolates of this bacterium (26).

Rhamnolipids are glycolipids produced by *P. aeruginosa* which reduce water surface tension and emulsify oil. These compounds are biodegradable and have potential industrial and environmental applications (14, 17). Recently, rhamnolipids have been found to have antagonistic effects on economically important zoosporic plant pathogens, thus opening up their use as biocontrol agents (29). The rhamnolipids produced by *P. aeruginosa* in liquid cultures (Fig. 1) are mainly rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (monorhamnolipid) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (dirhamnolipid). Rhamnolipid biosynthesis proceeds through two rhamnose transfers from TDP-L-rhamnose (3). For the synthesis of monorhamnolipid, the enzyme rhamnosyltransferase 1 (Rt 1) catalyzes the rhamnose transfer to β-hydroxydecanoyl-β-hydroxydecanoate, while Rt 2 synthesizes dirhamnolipid from TDP-L-rhamnose and monorhamnolipid. Genes coding for biosynthesis, regulation, and induction of Rt 1 enzyme are organized in tandem in the *rhlABRI* gene cluster around min 38 of the *P. aeruginosa* chromosome (20). The genes encoding Rt 2 have yet to be described.

Polyhydroxyalkanoates (PHAs) are bacterial storage compounds, which are synthesized by the polymerization of β-hydroxyacids by the PHA synthases (PhaC), with the coenzyme A (CoA)-linked fatty acids as substrates (Fig. 1) (31). The NADPH-dependent β-ketoacyl-CoA reductase (PhaB) is responsible for the reduction step in the production of the β-hydroxyacids. These storage compounds are intracellularly deposited as granules in many species. *P. aeruginosa* mainly produces PHAs consisting of medium-chain-length polymers, mainly poly-β-hydroxydecanoate (30).

The fatty acid synthetase system of *Escherichia coli* as well as that of most bacteria and plants is a dissociated fatty acid type of system (i.e., different reactions are catalyzed by separate proteins encoded by separate genes) (7). This biosynthetic pathway has been widely studied at the molecular level in *E. coli* and is encoded by a cluster of genes called *fab* genes which have been cloned and sequenced. As shown in Fig. 1, each round of elongation requires four chemical reactions.

* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Morelos 62250, Mexico. Phone: (52) (73) 291634. Fax: (52) (73) 172388. E-mail: gloria@ibt.unam.mx.

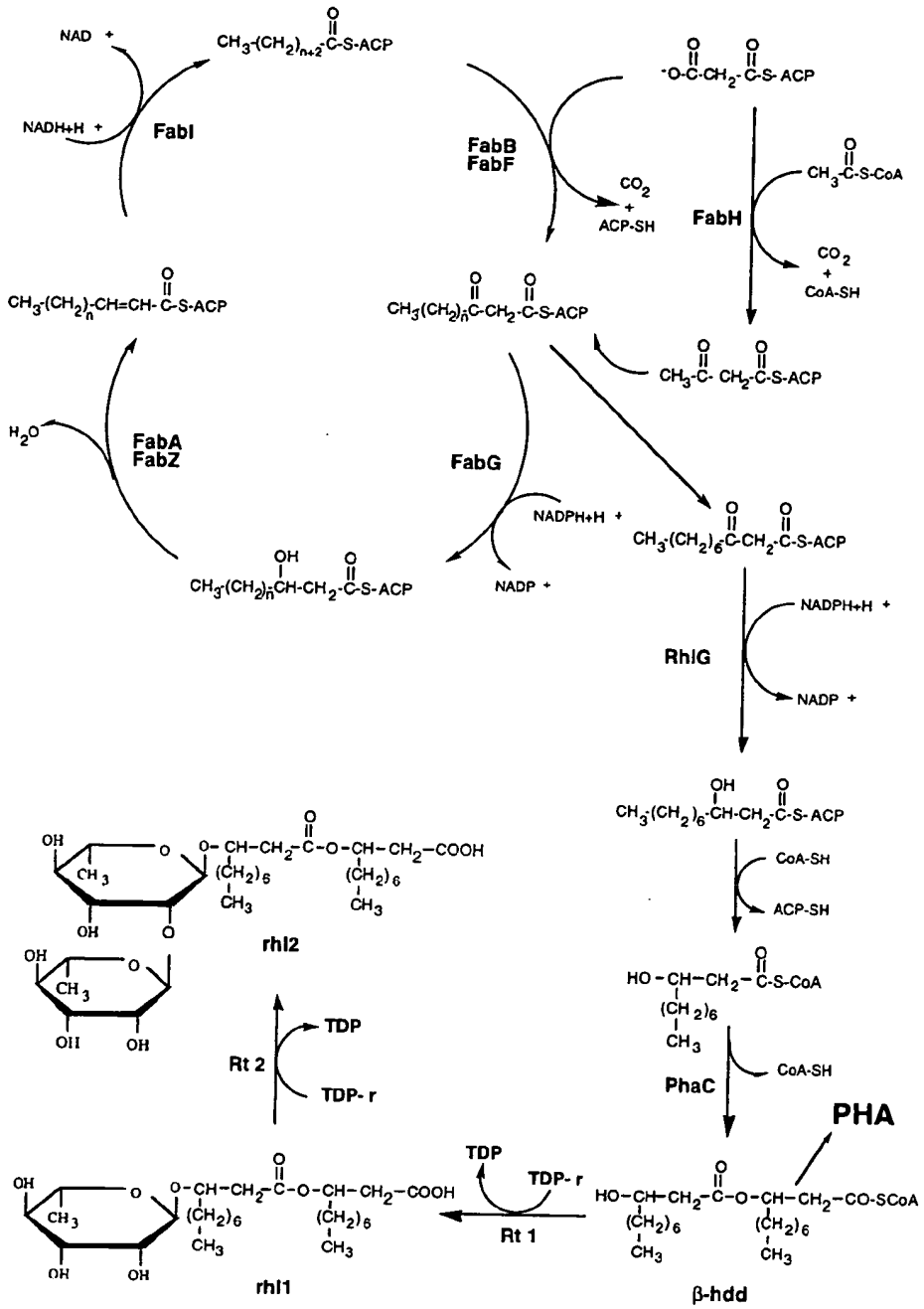

FIG. 1. Schematic representation of the fatty acid biosynthetic pathway showing the deduced role of the RhlG protein in the production of rhamnolipids and PHAs. Initiation of the fatty acid biosynthetic cycle, catalyzed by FabH, requires acetyl-CoA and malonyl-ACP to form aceto-acetyl-ACP. Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by FabB and FabF. In the second step, the resulting β-ketoester is reduced to a β-hydroxyacyl-ACP by FabG. The third step in the cycle is catalyzed by either FabA or FabZ. The fourth and final step is the conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by FabI. TDP-r, thymidine-diphospho-L-rhamnose; PhaC, PHA synthase; rhl 1, monorhamnolipid; rhl 2, dirhamnolipid; β-hdd, β-hydroxydecanoyl-β-hydroxydecanoate.

Initiation requires acetyl-CoA and malonyl-acyl carrier protein (ACP) to form aceto-acetyl-ACP. The first cycle is initiated by Kas III (FabH). Subsequent cycles are initiated by condensation of malonyl-ACP with acyl-ACP, catalyzed by Kas I (FabB) and Kas II (FabF). In the second step, the resulting β-ketoester is reduced to a β-hydroxyacyl-ACP by a single, NADPH-dependent β-ketoacyl-ACP reductase (FabG). The third step in the cycle is catalyzed by either the *fabA*- or *fabZ*-encoded β-hydroxyacyl-ACP dehydratases. The fourth and final step is the conversion of *trans*-2-enoyl-ACP to acyl-ACP, a reaction catalyzed by a single NADH-dependent enoyl-ACP reductase (FabI).

Recently the complete *P. aeruginosa fab* gene cluster sequence was deposited in the GenBank database (accession no.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
P. aeruginosa		
W51D	Strain able to degrade surfactants	28
W51D-10	W51D *rhlG*::Tc mutant	This work
PAO1	Wild-type strain	B. H. Holloway[b]
ACP5	PAO1 *rhlG*::Tc mutant	This work
PAO R1	PAO1 *lasR*::Tc mutant	12
C. violaceum		
ATCC 31532	Wild-type strain	16
CV026	ATCC 31532 nonpigmented mutant	16
Plasmids		
pJQ200mp18	Cloning vector Gm^r, unable to replicate in *Pseudomonas*	25
pJC1	pJQ200mp18 with a 600-bp *rhlG* internal fragment	This work
pJC2	pJC1 with a Tc^r cassette cloned into the single *Sma*I site of *rhlG*	This work
pUCP20	pUC19-derived *E. coli-Pseudomonas* shuttle vector, Cb^r	33
pJC3	pUCP20 with the PAO1 *rhlG* gene obtained by PCR	This work
pJC4	pUCP20 with 7 kb of W51D DNA, including the *rhlG* gene	This work

[a] The abbreviations used represent resistance to carbenicillin (Cb^r), gentamicin (Gm^r), and tetracycline (Tc^r).
[b] Monash University, Clayton, Victoria, Australia.

U91631). In addition, the *P. aeruginosa fabA* and *fabB* genes have been characterized (15). The objective of this work is to present evidence for the existence of a *P. aeruginosa* gene (*rhlG*) encoding a FabG homolog which is specifically involved in the synthesis of the β-hydroxyacid moiety of rhamnolipids. There have been no previous reports on the nature of the enzymes involved in the synthesis of the β-hydroxyacid moiety of rhamnolipids, and it has been assumed that they are the same proteins involved in fatty acid synthesis. Evidence is also presented suggesting that RhlG has a role in PHA synthesis.

MATERIALS AND METHODS

Microbiological procedures. The bacterial strains and plasmids used in this work are shown in Table 1. *P. aeruginosa* strains were routinely grown on Luria-Bertani medium (LB), *Pseudomonas* isolation agar (PIA [Difco]), or PPGAS (the phosphate-limited medium designed for rhamnolipid production) (34) at 29°C. M9 minimal medium supplemented with 0.05% NH_4Cl and gluconate 0.2% (MM + gluconate) was used to induce the production of PHAs. The antibiotic concentrations used for *P. aeruginosa* PAO1 and W51D, respectively, were as follows: carbenicillin, 250 and 50 μg/ml; gentamicin, 250 and 30 μg/ml; and tetracycline, 150 and 50 μg/ml.

Exoproducts and PHA determination. Pyocyanine was extracted with chloroform from the culture supernatant and determined by A_{690} as described previously (6). Protease production was measured by halo formation in LB plates containing 1% skim milk and inoculated with 20 μl of a saturated liquid culture. Total rhamnolipid concentration was determined from culture supernatants of cells grown on PPGAS medium at 29°C for 48 h by measuring the rhamnose concentration after acid hydrolysis by the orcinol method (4). The production of butanoyl-homoserine lactone (PAI-2) by different *P. aeruginosa* strains was determined by using the biosensor developed for the detection of small-chain *N*-acyl-homoserine lactones based on violacein production by *Chromobacterium violaceum* mutant strain CV026 (16). The wild-type *C. violaceum* strain ATCC 31532 produces violacein induced by the autoinducer *N*-hexanoyl-L-homoserine lactone, while mutant CV026 only produces this pigment when given medium supplemented with this autoinducer or related compounds, such as the *P. aeruginosa* PAI-2 autoinducer. PHA was determined after 24 h of growth under nitrogen-deprived conditions (30). Cells were harvested by centrifugation and washed with 100 mM Tris–100 mM NaCl buffer (pH 7). Cells were ruptured by sonication, and the extract was digested with 1.8% sodium hypochlorite for 1 h. After centrifugation, the pellet was washed twice with ethanol and once with acetone. The PHA concentration is expressed as milligrams of PHA per milligram of protein.

Fatty acid analysis. Total cell lipids were extracted by the method of Folch et al. (10). Briefly, 1 ml of the culture was washed twice and then brought back to the original volume. The following reagents were added with vortexing after each addition: 2 ml of 2:1 methanol-chloroform, 1 ml of 1 N KCl acidified with 0.1 N HCl, and 1 ml of chloroform. In some samples, a white emulsion phase formed between the aqueous and organic phases. In this case, the sample was placed in the refrigerator overnight to allow the emulsion phase to settle. The lower phase (chloroform) was removed and evaporated at 45°C under a nitrogen stream. The fatty acids were analyzed by gas chromatography after methyl esterification (18). Chloroform (0.5 ml) was added, and the sample was vortexed. Two milliliters of BF_3-methanol was added, and the mixture was heated at 80°C for 1 h in an airtight Teflon sealed screw-cap tube (18). The resulting fatty acid methyl esters (FAMEs) were extracted three times with 1 ml of hexane, and the three fractions were combined. Finally, the hexane was evaporated at 45°C under a nitrogen stream, and the FAMEs were brought to a concentration of 200 μl with chloroform.

Electron microscopy. PHA production by different *P. aeruginosa* strains was visualized by electron microscopy. Cells were treated for electron microscopic observation as follows. They were washed three times with phosphate buffer at pH 7.2, fixed with 2% glutaraldehyde for 2 h, and washed with phosphate buffer. Further fixation with 2% osmium tetroxide for 2 h was done; all of these procedures were carried out at 4°C. Fixed cells were washed and then dehydrated by passage through a graded ethanol series. After exposure to propylene oxide, samples were placed in L. R. White resin as recommended by the manufacturer. Ultrathin sections were incubated with uranyl acetate, washed with distilled water, treated with lead citrate, washed again, and observed.

Nucleic acid procedures. DNA isolation, cloning and sequencing, Southern and Northern blotting, and nick translation procedures were carried out as described previously (27). RNA was isolated with the RNaid PLUS kit (BIO101, Inc.). Primer extension analysis was done with two primers (R3 and R4 [Fig. 2]), both located in the 5′ region of the *rhlG* gene from *P. aeruginosa* PAO1 (*Pseudomonas* Genome Project contig 1780). The templates used for sequencing reactions were obtained by PCR of total DNA from *P. aeruginosa* PAO1 with the oligonucleotides L1 and R3 or R4 (Fig. 2). The sequencing reactions were done with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Inc.).

Genetic manipulations. *P. aeruginosa* matings (34) and transformation (21) were done as reported previously. The PAO1 and W51D *rhlG*::Tc mutants (ACP5 and W51D-10, respectively [Table 1]) were constructed by selection of double recombination events with plasmid pJC2. This plasmid is a derivative of plasmid pJC1, which contains a 600-bp *rhlG* internal fragment from *P. aeruginosa* W51D. A 1.4-kb tetracycline resistance gene from plasmid pBSL141Tc (1) was cloned on the unique *Sma*I site of the *rhlG* fragment contained in plasmid pJC1, rendering plasmid pJC2 (Table 1). The W51D *rhlG* internal fragment was obtained by PCR with the oligonucleotides L2′ (CGAACTCTGCAGGTACGGC GAGTGCATCGG) and R2′ (GATGCTGCAGATGTTGCCGGTCATGTAG GC) (corresponding to the positions in the PAO1 *rhlG* gene of the L2 and R2 oligonucleotides shown in Fig. 2), with the recognition site for the *Pst*I endonuclease incorporated on the flanking ends of both of them. Plasmid pJC3 is a pUCP20 (33) derivative containing the PAO1 *rhlG* gene obtained by PCR with oligonucleotides L1 and R1 (Fig. 2). Plasmid pJC4 is a pUCP20 derivative containing a 7-kb *Eco*RI W51D DNA fragment which includes the *rhlG* gene. The sequence of the L1 oligonucleotide is not present in the W51D *rhlG* gene region.

Computer analysis of the DNA and protein sequences. Computer analyses of the sequences were carried out by using the GENE WORKS program (IntelliGenetics, Inc.) and the University of Wisconsin Genetics Computer Group (UWGCG) programs. The sequences of different *P. aeruginosa* PAO1 contigs were obtained from the *Pseudomonas* Genome Project web site (http://www.pseudomonas.com).

Nucleotide sequence accession number. The sequence of the W51D *rhlG* gene has been deposited in the GenBank database under accession no. AF052586.

RESULTS AND DISCUSSION

Identification and sequencing of the *P. aeruginosa* W51D *rhlG* gene. *P. aeruginosa* W51D is a bacterium which is able to degrade at least 70% of a branched-chain alkylbenzene sulfonate mixture and is resistant to high concentrations of this surfactant (28). In order to study the W51D surfactant catabolic pathway, we have isolated several transposon mutants affected in the degradation of presumed intermediates of the surfactant degradation (unpublished results). During the DNA sequencing of a mutant unable to degrade citronellol, we detected a linked open reading frame (ORF) which was homologous to the *E. coli fabG* gene, having 36% amino acid se-

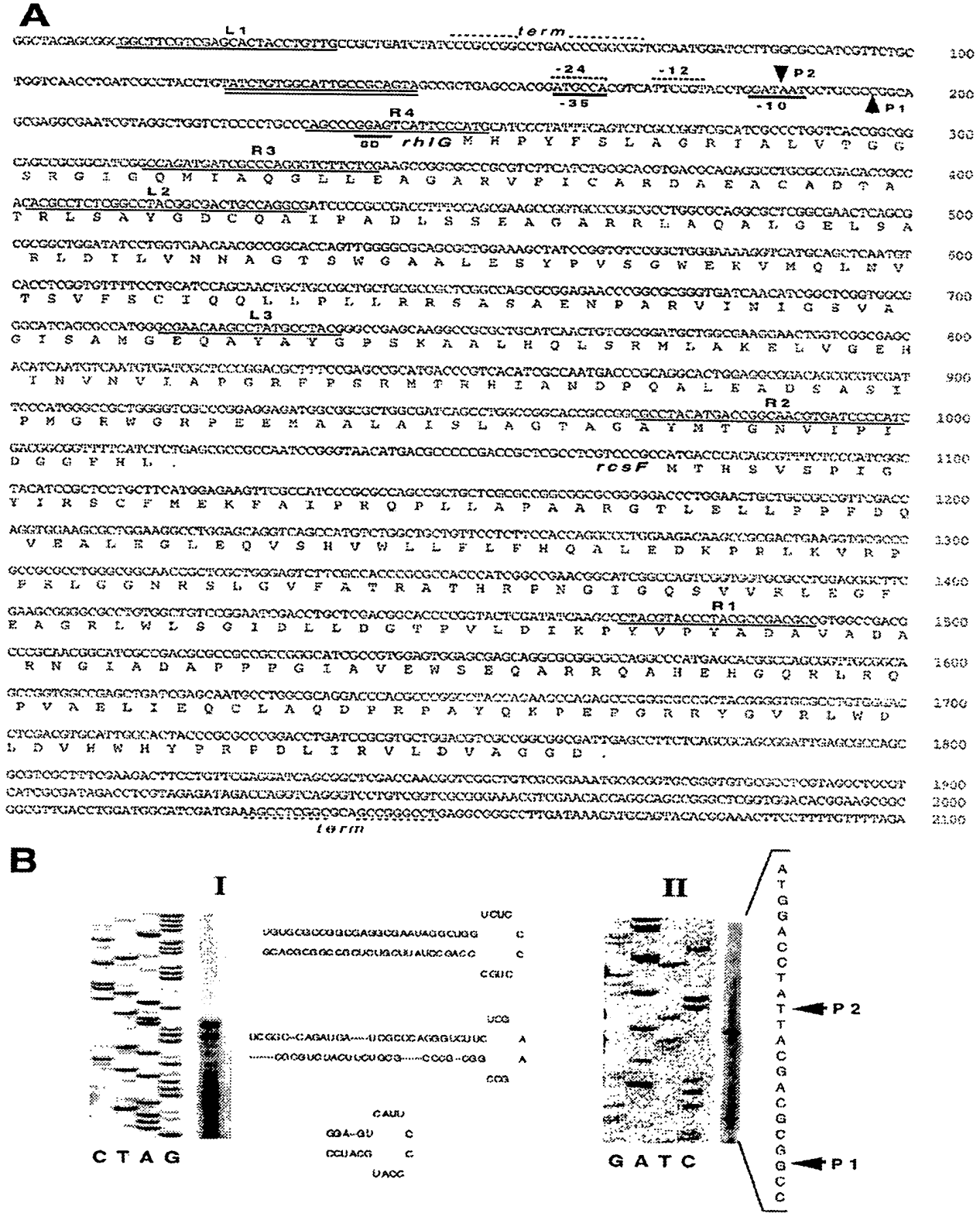

FIG. 2. Characterization of the transcription arrangement of the *P. aeruginosa* PAO1 *rhlG* and *rcsF* genes. (A) Nucleotide sequence of the genes and regulatory sequences. The sequence and position of the oligonucleotides used during this work are shown in the figure and identified as Ln or Rn, depending on their polarity (L oligonucleotides amplify the sequence from 5′ to 3′, and R oligonucleotides have the opposite polarity). The sequence corresponding to the lux box is double underlined. Arrows indicate the two transcription start sites detected (P1 and P2). SD (Shine-Dalgarno) indicates the ribosome binding site sequence for mRNA translation. The sequences corresponding to putative transcriptional termination sites (term) are shown. (B) Primer extension analysis of the *rhlG* gene with two different oligonucleotides as primers. In panel BI, the primer extension analysis was done with oligonucleotide R3 and revealed the existence of the mRNA secondary structures shown. In panel BII, the oligonucleotide R4 was used, and two transcription start points indicated as P1 and P2 were found.

quence identity along the entire length. This ORF, called *rhlG*, has the characteristic codon usage and bias of its GC composition in the third position of each codon of the *Pseudomonas* genes (32). The alignment of the protein deduced from the sequence of the *rhlG* gene with the proteins deposited in the GenBank database confirmed the presence of the characteristic signature for NADPH binding, as well as the characteristic motifs of dehydrogenases. As shown in Fig. 3, these sequences are conserved in all of the sequenced bacterial and plant FabG proteins. However, the chromosomal region surrounding this *fabG* homolog did not show the presence of other *fab* genes, as has been reported for *P. aeruginosa* PAO1 and *E. coli*.

Another ORF encoding a protein with a sequence 44% identical to that of *E. coli* RcsF, which is involved in regulation of capsular production (13), was detected downstream of *rhlG*. This genetic arrangement and the fact that a *fabG* gene has been already described in *P. aeruginosa* PAO1 led us to the hypothesis that this is a novel gene which encoded a second functional NADPH-dependent β-ketoacyl reductase. In order to test this hypothesis, we constructed an *rhlG*::Tc mutant according to the strategy shown in Fig. 4. The inactivation of the W51D *rhlG* gene did not produce a fatty acid auxotrophy or a decrease in growth rate (data not shown). These results showed that the RhlG protein is not responsible for the total cellular fatty acid synthesis, so a FabG protein should also exist in strain W51D. However, this evidence was not enough to determine the expression and functionality of the *rhlG* gene product.

Identification of the *rhlG* gene in the *P. aeruginosa* PAO1 genome. We decided to study the functionality of the RhlG protein in the *P. aeruginosa* PAO1 strain for two reasons: approximately 95% of its genome has been sequenced (http://www.pseudomonas.com), and the existence of the *fabG* gene had already been reported in this strain (GenBank database accession no. U91631). We identified the PAO1 *rhlG* gene in contig 1780 of the *Pseudomonas* Genome Project, showing the characteristic codon usage and bias of GC composition in the third position of each codon of the *Pseudomonas* genes (32). The genetic arrangement of PAO1 is similar to that of strain W51D, in which the *rcsF* gene is downstream (Fig. 2). The deduced PAO1 RhlG protein consists of 256 amino acids, with a predicted molecular mass of 26,813 Da and has amino acids 54% identical to those of the W51D RhlG protein (Fig. 3). The great divergence between both *rhlG* genes is mainly due to differences in the sequences at their 5′ ends. If the sequences are compared after deletion of the first 52 amino acids of the PAO1 RhlG protein and 112 amino acids of the corresponding protein in W51D, they have 91% identical amino acid sequences. Furthermore, both proteins contain the motifs important for their putative catalytic capabilities (Fig. 3). The difference between the amino-terminal sequences of PAO1 and W51D RhlG proteins is striking, considering that both strains belong to the same species. The significance of this variability is not clear to us. The DNA sequence of the first 300 nucleotides of the PAO1 *rhlG* 5′ region (Fig. 2) was confirmed by us, and we found only three differences. This result rules out the possibility that the divergence between the *rhlG* genes is due to major inaccuracies in the reported sequence in contig 1780.

We confirmed that the *rhlG* genes were conserved and that *rcsF* was present downstream in PAO1 and W51D strains by PCR amplification of total DNA (Fig. 4C). The following oligonucleotides were used as primers: L2 or L2′ (the oligonucleotide corresponding to the W51D *rhlG* gene sequence in the same region) and R2, L2 or L2′ and R1, and L3 and R1 (Fig. 2A). The amplified product was a DNA band of the same size from either strain (Fig. 4C), thus validating the high degree of homology between *rhlG* genes and the conservation of the genetic arrangement inferred from the analysis of the sequence obtained from the *Pseudomonas* Genome Project in contig 1780.

The sequences of the PAO1 *fabG* gene from contig 1761 of the *Pseudomonas* Genome Project and GenBank (accession no. U91631) were compared. The two PAO1 *fabG* gene DNA sequences are not identical. This inconsistency may result from inaccuracies in the sequence of the *Pseudomonas* Genome Project. We compared both PAO1 FabG protein sequences to the deduced protein sequences of the PAO1 RhlG protein and found the amino acids were 33 and 34% identical, respectively (Fig. 3). This is further evidence that RhlG is an NADPH-dependent β-ketoacyl reductase. The PAO1 RhlG protein also aligned with FabG proteins of different origin, as well as with PhaB proteins from *Alcaligenes* sp. strain SH-69 and *Acinetobacter* sp. strain RA3849 (Fig. 3). This result is not surprising, since the PhaB proteins are NADPH-dependent acetoacetyl reductases which participate in PHA synthesis. The significance of the RhlG homology with PhaB proteins is discussed below.

Expression of the *rhlG* gene in *P. aeruginosa* PAO1. We carried out primer extension experiments to determine whether the *rhlG* was expressed in strain PAO1 grown for 48 h on PPGAS, a medium designed to increase rhamnolipid production (34). Two oligonucleotides derived from the DNA sequence reported in contig 1780 corresponding to the 5′ end of the *rhlG* gene were used as primers (Fig. 2). These experiments revealed the presence of a specific *rhlG* mRNA, confirming that the gene is expressed under these culture conditions (Fig. 2BI and BII). When the R3 oligonucleotide was used, the extension was aborted very near the putative RhlG protein start codon, suggesting the existence of an mRNA region with a secondary structure that prevented DNA polymerization by reverse transcriptase beyond this point (Fig. 2BI). The DNA sequence within this region predicted the formation of several loops in the mRNA (Fig. 2BI), which could play a role in the regulation of the *rhlG* gene expression at the posttranscriptional level.

Two mRNA start sites were observed when the oligonucleotide R4 was used as a primer in extension experiments (Fig. 2BII). R4 is complementary to the mRNA sequence in which the extension of the primer was aborted with oligonucleotide R3 (Fig. 2A). The most frequent mRNA start site seems to be transcribed from a putative σ^{54} type of promoter, although the −12/−24 regions do not present all the elements which have been claimed to be important in these promoters. A similar situation has been found in the *rhlAB* σ^{54} promoter (24). The second, less abundant mRNA start site is a typical σ^{70} type of promoter. These two promoters overlap at their respective −24 and −35 regions. Between nucleotides −43 and −63 (with respect to the putative σ^{54} promoter), the sequence ATCTG TGGCATTGCCGCAGTA corresponding to a "lux box" is present (Fig. 2A). The presence of this regulatory sequence strongly suggests that the *rhlG* gene is regulated at the transcriptional level by one of the two LuxR homologs forming part of the quorum-sensing type of response in *P. aeruginosa*, LasR or RhlR. The characteristics of the *rhlG* promoter region (two promoters, one of which is a noncanonical σ^{54} type of promoter, and the presence of a lux box) are very similar to those present in the promoter region of the *rhlAB* operon, which encodes the Rt 1 enzyme (24). In the case of this key enzyme for rhamnolipid biosynthesis, RhlR positively regulates its transcription (20), and the alternative sigma factor σ^{54} is involved in its expression (24). As will be shown later, RhlG protein is involved in the synthesis of one of the rhamnolipid

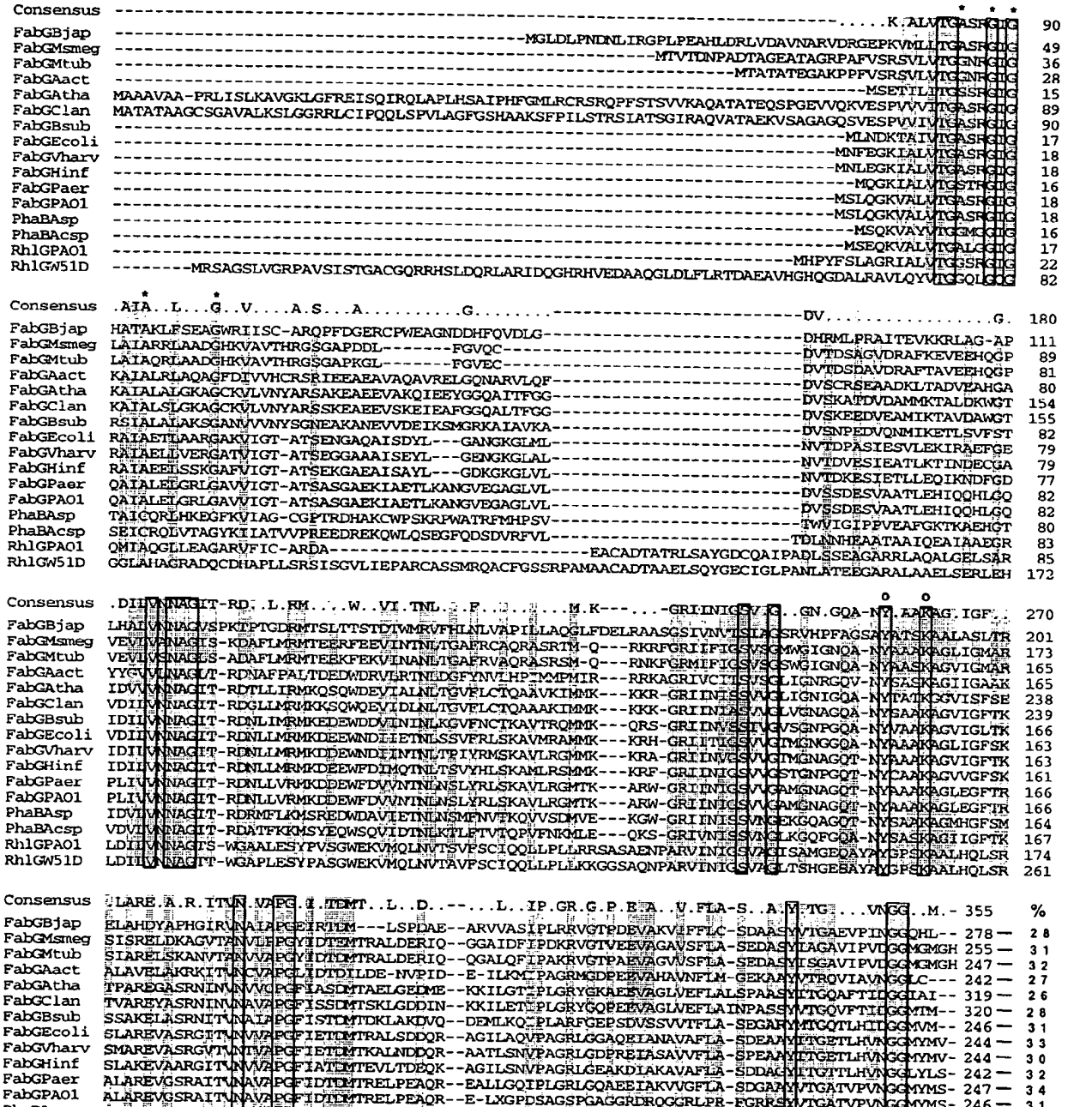

FIG. 3. Multiple alignment of the RhlG deduced amino acid sequence with different NADPH-dependent β-ketoacyl-ACP reductase (FabG) and NADPH-dependent ketoacyl-CoA reductase (PhaB) proteins. Residues within rectangles correspond to identical amino acids, and those shaded are conserved among most of the proteins analyzed. The percentage of identity of the different proteins with PAO1 RhlG is shown in the bottom right column of the figure. Asterisks mark the residues which form the NADPH binding signature, and circles show the amino acids conserved in dehydrogenases. FabGBjap, FabG from *Bradyrhizobium japonicum*; FabGMsmeg, FabG from *Mycobacterium smegmatis*; FabGMtub, FabG from *Mycobacterium tuberculosis*; FabGAact, FabG from *Actinobacillus actinomycetemcomitans*; FabGAtha, FabG from *Arabidopsis thaliana*; FabGClan, FabG from *Cuphea lanceolata*; FabGBsub, FabG from *Bacillus subtilis*; FabGEcoli, FabG from *E. coli*; FabGVharv, FabG from *Vibrio harveyi*; FabGHinf, FabG from *Haemophilus influenzae*; FabGPaer, FabG from *P. aeruginosa* (GenBank database accession no. U91631); FabGPAO1, FabG from *P. aeruginosa* PAO1 (contig 1761); PhaBAsp, PhaB from *Alcaligenes* sp. strain SH69; PhaBAcsp, PhaB from *Acinetobacter* sp. strain RA3849; RhlGPAO1, RhlG from *P. aeruginosa* PAO1 (contig 1780); RhlGW51D, RhlG from *P. aeruginosa* W51D (GenBank database accession no. AF052586).

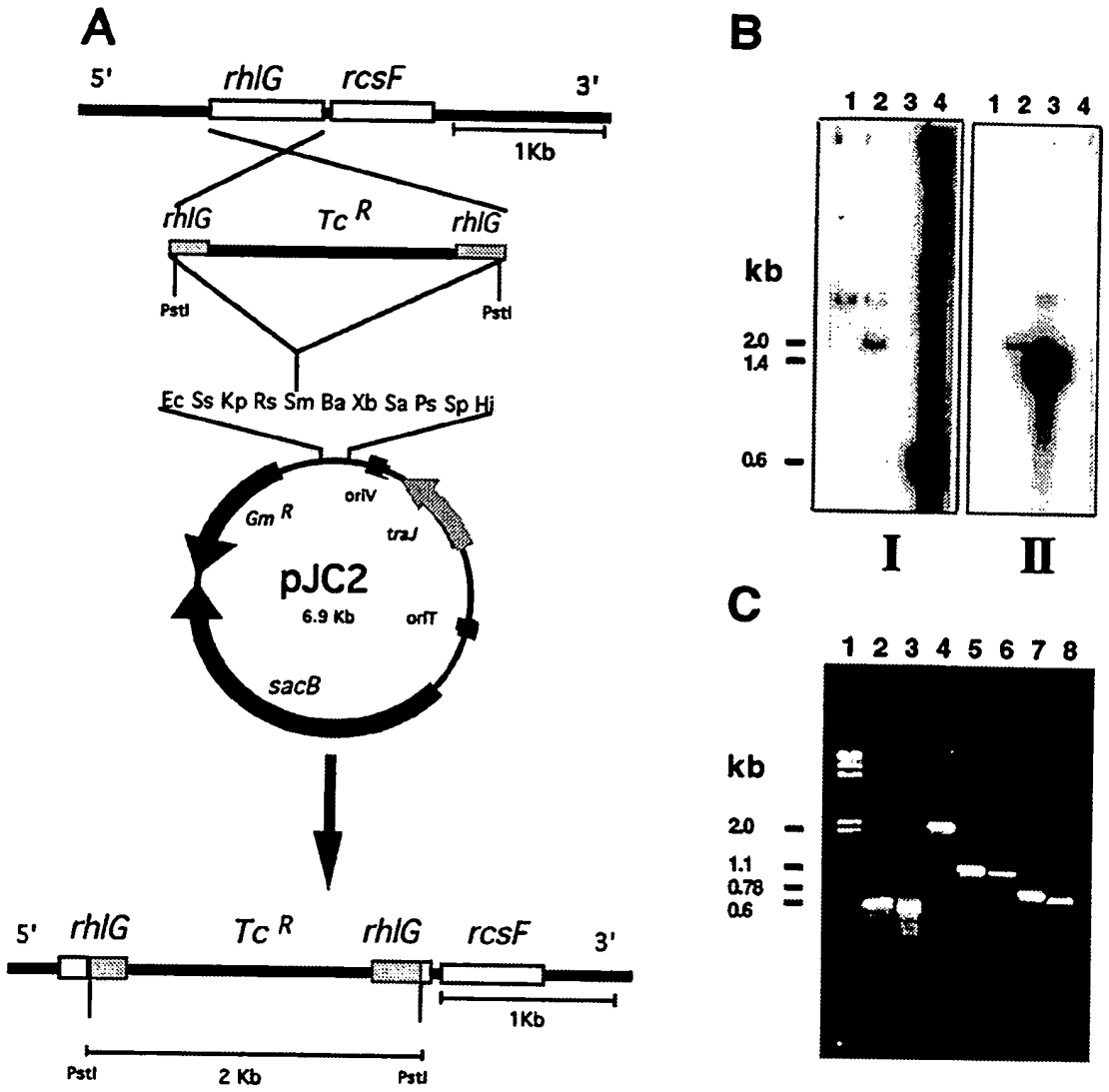

FIG. 4. Molecular characterization of the PAO1 *rhlG* mutant ACP5. (A) Schematic representation of the strategy to construct the *rhlG* mutants (ACP5 and W51D-10). (B) Southern blotting hybridization with the 600-bp insert of plasmid pJC1 (I) and the 1.4-kb Tc^r resistance cassette (II) used as probes. Lanes correspond to DNA samples digested with *PstI* endonuclease from the PAO1 genome (lane 1), the ACP5 genome (lane 2), the Tc^r cassette (lane 3), and the PCR product of the amplification of the W51D genome with oligonucleotides L2' and R2' (lane 4). (C) Amplification by PCR with different oligonucleotides specific for the *rhlG* and *rcsF* genes. Lanes correspond to the following DNA samples: 1, λ phage genome digested with *HindIII*; 2, PCR product with W51D DNA as a template and the L2' and R2 oligonucleotides as primers; 3, PCR product with PAO1 DNA as a template and the L2 and R2 oligonucleotides as primers; 4, PCR product with ACP5 DNA as a template and the L2 and R2 oligonucleotides as primers; 5, PCR product with W51D DNA as a template and the L2' and R1 oligonucleotides as primers; 6, PCR product with PAO1 DNA as a template and the L2 and R1 oligonucleotides as primers; 7, PCR product with W51D DNA as a template and the L3 and R1 oligonucleotides as primers; and 8, PCR product with PAO1 DNA as a template and the L3 and R1 oligonucleotides as primers.

precursors, so it is likely that the structural similarity between the promoter regions of the *rhlG* gene and the *rhlAB* operon reflects that they are subject to similar genetic regulation. This possibility was examined further (see below).

Construction of a *P. aeruginosa* PAO1 *rhlG*::Tc mutant. The high degree of similarity of the PAO1 and W51D *rhlG* genes, excluding their 5' ends (Fig. 3), enabled us to construct a PAO1 *rhlG*::Tc mutant (ACP5 [Table 1]). Plasmid pJC2, which contains an *rhlG* internal fragment from strain W51D with a Tc^r cassette insertion, was transferred by transformation to strain PAO1, and Tc^r Gm^s transformants which were putative double recombinants carrying an interrupted *rhlG* gene were selected (Fig. 4). One of these transformants is the ACP5 mutant (Table 1), which indeed seems to be the product of a double recombination event in which the *rhlG* gene is interrupted by the Tc^r cassette; this conclusion is drawn from the analysis by Southern blot hybridization and PCR amplification as shown in Fig. 4B and C, respectively. The Southern blot hybridization analysis (Fig. 4B) shows that mutant ACP5 contains, as expected, a 2-kb *PstI* fragment with homology with both the *rhlG* gene and the Tc^r cassette (lanes 2 in Fig. 4BI and BII) and that this fragment is not present in the PAO1 genome (lanes 1, Fig. 4BI and BII). Unexpectedly, however, the ACP5 DNA retained hybridization with the 3.2-kb *PstI* *rhlG* homologous band. This result can be explained by the presence of heterogeneity in the chromosomes of strain ACP5, in which

TABLE 2. Production of rhamnolipids and pyocyanine by mutant ACP5 and its parental strain, PAO1

Strain	Concn (%) of[a]:		
	Rhamnolipid	Pyocyanine	PHA
PAO1	150 ± 15 (100)	0.59 (100)	311 ± 13 (100)
ACP5	<2	0.24 (40.6)	87 ± 4 (27.9)
ACP5/pJC3	125 ± 25 (83.3)	0.60 (101.6)	243 ± 30 (78)
ACP5/pJC4	146 ± 10 (97.3)	0.64 (108.4)	305 ± 5 (98)
PAO R1	<2	<0.05	ND[b]

[a] Rhamnolipid concentration is expressed as micrograms of rhamnose in rhamnolipids per milliliter of culture. The concentration of pyocyanine is expressed as the A_{690} of the chloroform-extracted culture supernatant. PHA was measured after 24 h of growth on MM + gluconate and is expressed as milligrams of PHA per milligram of protein.
[b] ND, not determined.

not all of the *rhlG* copies contain a Tc^r cassette, or by the presence in the PAO1 chromosome of an *rhlG* homolog (probably *fabG*), which gives a hybridization signal of the same size when DNA is digested with *Pst*I. In order to distinguish between these possibilities, PCR was performed in which *rhlG*-specific oligonucleotides (L2 and R2 in Fig. 2) were used to amplify the PAO1 and ACP5 genomes. We found that the expected 600-bp DNA fragment is amplified from PAO1, while a single 2-kb band is amplified from the ACP5 genome (Fig. 4C, lanes 3 and 4), these results clearly show that all of the *rhlG* gene copies in ACP5 contain a 1.4-kb insert (the Tc^r cassette), so the most likely explanation is that we are detecting an *rhlG* homologous gene by Southern blot hybridization, probably *fabG*.

Effect of the *rhlG* inactivation in *P. aeruginosa* PAO1. Mutant ACP5 does not have a fatty acid auxotrophy, grows at the same rate as its PAO1 parental strain, and does not show any significant change in its total lipid profile. Furthermore, the total lipid profiles of the parent and mutant strains were identical (data not shown). This suggests that there must be a functional FabG protein that is responsible for the synthesis of total cellular lipids and other essential products which contain a fatty acid moiety, such as the lipid A molecule (9).

P. aeruginosa produces different secondary metabolites which contain a lipid moiety, such as the autoinducers PAI-1 and PAI-2, as well as rhamnolipids and PHAs. Therefore, we investigated whether the production of some of these compounds was affected by the cassette insertion in the *rhlG* gene (mutant ACP5). Mutants affected in the production of any of the autoinducers are defective in total protease production (2, 22). We used this phenotype as a criterion to evaluate autoinducer production. It was found that mutant ACP5 has the same proteolytic activity as the PAO1 parental strain (data not shown), suggesting that autoinducer production is not affected. Rhamnolipid production in mutant ACP5 is completely abrogated (Table 2), suggesting that the RhlG protein is involved in the reaction leading to the production of the β-hydroxydecanoyl precursor of rhamnolipids (Fig. 1). In order to obtain direct evidence of the involvement of RhlG protein in rhamnolipid production and to rule out that the phenotype of mutant ACP5 was due to a polar effect of the Tc^r cassette insertion in *rhlG* (and not to an inactivation of this gene), we complemented in *trans* the ACP5 mutant with plasmid pJC3, which contains the PAO1 *rhlG* gene (Table 1). The results obtained (Table 2) clearly show that the presence in *trans* of the *rhlG* gene is sufficient to restore the ACP5 capability to produce rhamnolipids.

It was apparent that mutant ACP5 produces lower levels of pigment than strain PAO1 (Table 2). It has been reported that

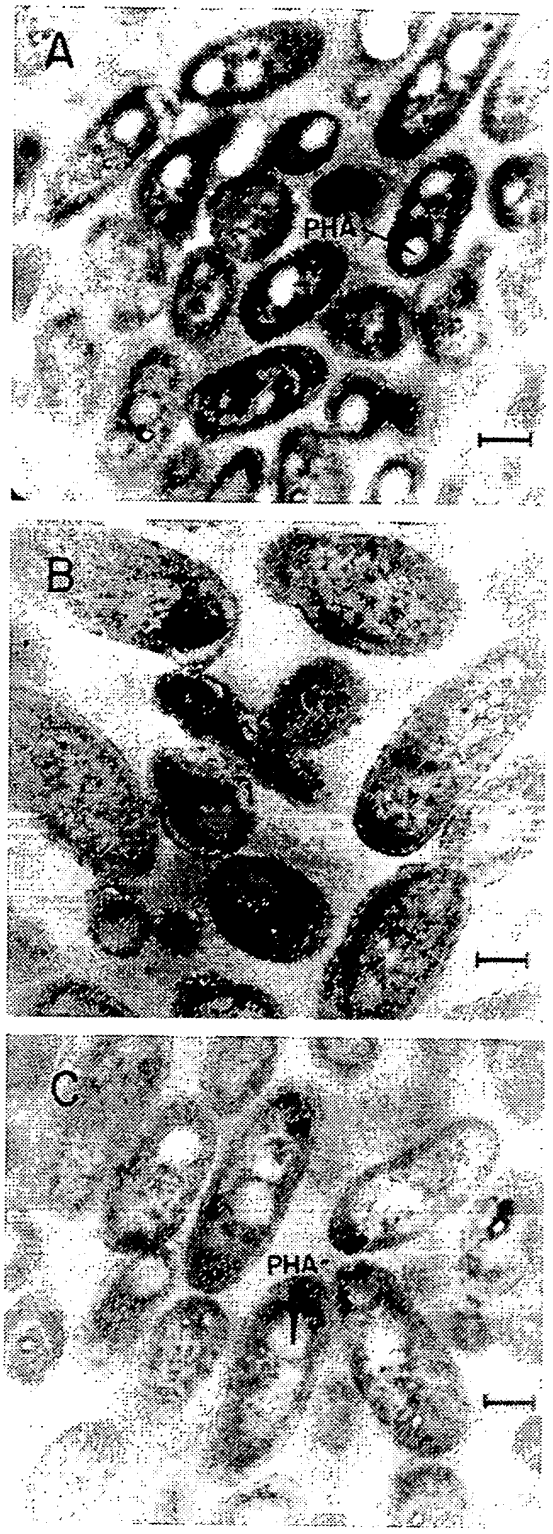

FIG. 5. Electron micrographs of the *P. aeruginosa* strains PAO1 (A), ACP5 (B), and ACP5/pJC4 (C) grown for 24 h on MM + gluconate. Some of the PHA granules are pointed out. Micrographs were taken at a ×20,000 magnification.

production of both rhamnolipids and pyocyanine is induced by PAI-2-mediated activation (20), so we measured PAI-2 production by using the *C. violaceum* CV026 biosensor (16). Mutant ACP5 produced PAI-2 autoinducer at levels similar to those produced by PAO1 (data not shown). At present, we do not have a clear explanation for the reduction in pigment formation by mutant ACP5, but both rhamnolipid production and pyocyanine production are restored upon introduction of a functional *rhlG* gene in plasmid pJC3 (Table 2).

P. aeruginosa is known to produce PHA by using fatty acids from the de novo synthesis as precursors (30). The production of total PHA is reduced in mutant ACP5 at 24 h of growth (Table 2), but reaches the same level as PAO1 after 96 h of growth (data not shown). This defect in PHA synthesis can be observed in the electron micrographs taken after 24 h of growth as a decrease in the number and size of granules in mutant ACP5 (Fig. 5). This deficiency is due to *rhlG* inactivation, since plasmid pJC3 restores PHA production (Table 2). These results suggest that RhlG plays a role in biosynthesis of fatty acids used as substrates for PHA production, but that it is not an absolute requirement.

These findings suggested the existence in PAO1 of other reductases involved in PHA production. As mentioned above, RhlG is homologous to PhaB proteins (Fig. 3), so we decided to search in the *Pseudomonas* Genome Project for PhaB homologs. We found that contig 983 contains an ORF coding for a protein with amino acids 30% identical to those of PhaB from *Acinetobacter* sp. strain RA3849 and 26% identical to those of RhlG from *P. aeruginosa* PAO1. It is very likely that the detected PHA synthesis in mutant ACP5 is due to the presence of an alternative pathway in which the reduction step is catalyzed by the putative acetoacetyl-CoA reductase encoded by the PAO1 *phbB* gene. Since this enzyme is expected to be used in polyhydroxybutanoyl synthesis, it would be interesting to determine whether the lengths of the fatty acid moiety of PHAs produced by mutant ACP5 are different from those of the PHAs produced by the wild-type strain, PAO1.

Plasmid pJC4, which contains 7 kb of the W51D chromosome, including the *rhlG* gene (Table 1), complemented in *trans* mutant ACP5 for rhamnolipid and pigment production and PHA synthesis (Table 2 and Fig. 5), suggesting that this gene has the same function in rhamnolipid and PHA synthesis in both *P. aeruginosa* strains.

Regulation of *rhlG* expression in *P. aeruginosa* PAO1. To obtain additional evidence in support of the involvement of the RhlG protein in rhamnolipid and PHA synthesis, the concentration of *rhlG* mRNA was quantified under different culture conditions. The maximum *rhlG* mRNA concentration is found under conditions in which rhamnolipid production is maximum (that is, the stationary phase of growth on PPGAS medium) (Table 3), but there is also considerable expression when bacteria are grown for 48 h on LB or MM + gluconate medium (Table 3). It is important to point out that in the latter medium, PAO1 also produced rhamnolipids (37 μg/ml after 24 h of growth). The level of expression of the *rhlG* gene in the exponential phase of growth was low under all culture conditions studied (Table 3). These results provide additional evidence of the involvement of RhlG in the production of secondary metabolites, such as rhamnolipids and PHA.

The DNA sequence of the *rhlG* promoter region suggested that the *rhlG* gene was regulated at the transcriptional level by one of the two LuxR homologs forming part of the quorum-sensing type of response in *P. aeruginosa*, LasR or RhlR. To obtain additional evidence in this respect, we determined the *rhlG* mRNA concentration of the PAO R1 strain (a PAO1 *lasR* mutant) grown on PPGAS medium. We used this mutant because it has been reported to be defective in both quorum-sensing regulatory circuits present in *P. aeruginosa* (12, 24). Table 2 shows that in agreement with these observations, PAO R1 lacks rhamnolipid and pyocyanine production when grown on PPGAS medium for 48 h. Unexpectedly, the level of PAO R1 *rhlG* mRNA concentration after 48 h of growth on PPGAS is only slightly lower than that of the wild-type PAO1 strain (Table 3), thus ruling out the direct involvement of LasR as the transcriptional activator of the *rhlG* gene. It is still possible that the RhlR protein activates *rhlG* transcription, since it has been shown that *rhlR* mRNA is expressed at a significant level in the PAO R1 mutant (24).

TABLE 3. Relative concentration of *rhlG* mRNA on different media and time of growth[a]

Strain	mRNA concn with growth time given[a]					
	PPGAS		LB		MM + gluconate	
	6 h	48 h	6 h	48 h	6 h	48 h
PAO1	45	417	35	209	24	248
PAO R1	—[b]	365	ND[c]	ND	ND	ND

[a] mRNA concentration is expressed as the ratio of the RNA hybridization detected by autoradiography scanning to total RNA concentration used in the experiment.
[b] —, not detected.
[c] ND, not determined.

This is the first report of the existence in *P. aeruginosa* of a ramification of the fatty acid biosynthetic pathway specifically involved in rhamnolipid production. Figure 1 shows the proposed role of RhlG protein in the rhamnolipid biosynthesis pathway. At present, we do not know whether the RhlG substrate is β-ketoacyl linked to ACP or to CoA. Our model (Fig. 1) shows the substrate to be β-ketoacyl-ACP, because most of the RhlG homologs are FabG-like enzymes (Fig. 3). We propose that CoA-β-hydroxyacids are the precursors of rhamnolipids, since the PHA synthases only use as a substrate the CoA-linked fatty acids (31), and the lipid moiety of rhamnolipids (β-hydroxydecanoyl-β-hydroxydecanoate) seems to be the product of these enzymes.

In summary, a new gene, *rhlG*, involved in rhamnolipid biosynthesis has been identified. The deduced RhlG protein shows significant sequence homology with numerous NADPH-dependent ketoacyl reductases. Complementation studies and measurement of the *rhlG* mRNA suggest that the RhlG protein is required for rhamnolipid biosynthesis and can be used in PHA production, but is not necessary for fatty acid synthesis.

ACKNOWLEDGMENTS

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AUTHOR: Lucy K M(a); Harshan K R(a)
AUTHOR ADDRESS: (a)Department of Anatomy, College of Veterinary and Animal
Sciences, Mannuthy, Thrissur, KER, 680 651, India**India
JOURNAL: Indian Journal of Poultry Science 33 (3):p326-328 %%%1998%%%
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ISSN: 0019-5529
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ABSTRACT: Histochemical studies on the quail oviduct were conducted to detect the presence of carbohydrates, lipids, acid and alkaline phosphatases (%%%ACP%%%&ALP). Lining epithelium showed goblet cells with
PAS-positive granules. Glycogen granules could be demonstrated in the ciliated columnar cells of the lining epithelium of isthmus which contributed to the carbohydrate portion of shell membrane. Sperm-host glands at the uterovaginal junction gave a positive reaction for %%%ACP%%%, ALP and lipids. %%%ACP%%% and ALP activities were detected in
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AUTHOR: Rodski Amanda(a); Petrolonis Anthony(a); Lewis Jennifer(a); Severs
Gregg(a); Kocylowski Laurene(a); Paik Moon-Kyu(a); Ruotolo Leonard(a); Terzaghi William B(a)
AUTHOR ADDRESS: (a)Dept. Biology, Wilkes University, Wilkes-Barre, PA, USA
**USA E-Mail: terzaghi@wilkes.edu
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